AD_____

Award Number: DAMD17-98-1-8080

TITLE: Analysis of hRad1, a Human G2 Checkpoint Control Gene

PRINCIPAL INVESTIGATOR: Blair D.A. Besley
Scott K. Davey, Ph.D.

CONTRACTING ORGANIZATION: Queen's University
Kingston, Ontario
Canada K7L 3N6

REPORT DATE: March 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2002	Annual Summary (1 Jun 00 - 1 Feb 02)

4. TITLE AND SUBTITLE
Analysis of hRad1, a Human G2 Checkpoint Control Gene

5. FUNDING NUMBERS
DAMD17-98-1-8080

6. AUTHOR(S)
Blair D.A. Besley
Scott K. Davey, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Queen's University
Kingston, Ontario
Canada K7L 3N6

E-Mail: 4bdab@qlink.queensu.ca

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

hRad1 and hRad9 are human homologs of proteins originally identified in fission yeast, which surmised to be responsible for the control of the G2/M checkpoint. We undertook studies to gain a further understanding of the complex and phosphorylation states of the hRad1 and hRad9 proteins, the results of which are discussed herein.

14. SUBJECT TERMS
breast cancer, hRad1 and hRad9 proteins, human homologs of proteins, fission yeast, G2/M checkpoint

15. NUMBER OF PAGES
129

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

This report describes the work of Blair Besley during his master's work in the Cancer Research Labs, as funded by award DAMD17-98-1-8080. This report contains a finished thesis describing studies detailing the complex state of the hRad9 checkpoint protein, and a novel cell-cycle specific phosphorylation of the hRad9 checkpoint protein.

Body

Please see the attached thesis in the appendix of this report for a detailed materials and methods, and all work carried out under award DAMD17-98-1-8080.

Key Research Accomplishments

- It was found that the hRad9 checkpoint protein exists in three distinct complexes, independent of cell cycle position.
- hRad9 is Phosphorylated in a cell cycle dependent manner, at G2/M of the cell cycle.
- The cell cycle phosphorylation of hRad9 occurs independent of DNA damage in the HeLa cervical cancer cells, but is DNA damage dependent in Karytoype-normal hTert-RPE1 cells.
- The cell cycle specific phosphorylation of hRad9 does not effect the binding of hRad9 to chromatin, and has an as-yet uncharacterized function.

Reportable Outcomes

Publications

- DNA damage-dependent and -independent phosphorylation of the hRad9 checkpoint protein.*J Biol Chem.* 2001 Nov 9;**276**(45):41898-905

Abstracts Presented

- **Besley BDA**, St. Onge RP, Park M, Davey SK. Cell Cycle Dependent and Independent Phosphorylation of Human Rad9. Proceedings of the Fourth Annual Meeting for Basic and Clinical Research Trainees in the Faculty of Health Sciences. Queen's University, Kingston ON

- St. Onge RP, **Besley BDA**, Davey SK. HRAD9 Phosphorylation and the DNA Damage Response. Proceedings of the Fourth Annual Meeting for Basic and Clinical Research Trainees in the Faculty of Health Sciences. Queen's University, Kingston ON

- **Besley BDA**, St. Onge RP, Greer DA, Udell CM, and Davey SK. Characterization and Disruption of the G2 Checkpoint Complex and the DNA Damage Dependent Interaction. Era of Hope Department of Defense Breast Cancer Research Program Meeting -- Proceedings. Atlanta GA, USA

- **Besley BDA**, Casselman R, Davey SK. Purification of Baculovirus Expressed G2 Checkpoint Complex. Proceedings of the Third Annual Meeting for Basic and Clinical Research Trainees in the Faculty of Health Sciences. Queen's University, Kingston ON

Degrees Granted

- Degree of Master of Science (Biochemistry) conferred to Blair DA Besley.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 45, Issue of November 9, pp. 41898–41905, 2001
Printed in U.S.A.

DNA Damage-dependent and -independent Phosphorylation of the hRad9 Checkpoint Protein*

Received for publication, June 5, 2001, and in revised form, August 22, 2001
Published, JBC Papers in Press, September 10, 2001, DOI 10.1074/jbc.M105152200

Robert P. St.Onge‡§¶, Blair D. A. Besley‡‖, Minwoo Park‡‖, Richard Casselman‡, and Scott Davey‡§‖‡‡§§**

From the ‡Cancer Research Laboratories and the Departments of §Pathology, ‖Biochemistry, and ‡‡Oncology, Queen's University, Kingston, Ontario K7L 3N6, Canada

Cell cycle checkpoints are regulatory mechanisms that maintain genomic integrity by preventing cell cycle progression when genetic anomalies are present. The hRad9 protein is the human homologue of *Schizosaccharomyces pombe* Rad9, a checkpoint protein required for preventing the onset of mitosis if DNA damage is present or if DNA replication is incomplete. Genetic and biochemical analyses indicate that hRad9 is a component of the checkpoint response in humans and has possible roles in regulating the cell cycle, apoptosis, and DNA repair. Previous studies indicate that hRad9 is modified by phosphorylation, both in the absence of exogenous stress and in response to various genotoxins. In this study, we report the mapping of several sites of constitutive phosphorylation of hRad9 to (S/T)PX(R/P) sequences near the C terminus of the protein. We also demonstrate that a serine to alanine mutation at residue 272 abrogates an ionizing radiation (IR)-induced phosphorylation of hRad9 and further show that phosphorylation at (S/T)P sites is not a prerequisite for IR-induced phosphorylation of serine 272. Finally, we report that hRad9 undergoes cell cycle-regulated hyper-phosphorylation in G_2/M that is enhanced by IR but distinct from that on serine 272. Unlike the IR-induced phosphorylation at serine 272, this event is dependent on serine 277 and threonine 292, two C-terminal (S/T)P sites in hRad9.

An organism's genome is under constant stress from a variety of endogenous and exogenous sources. Although low frequencies of genetic mutation are tolerated, contributing to genetic diversity, high frequencies are harmful and can lead to cancer (1). At the cellular level, eukaryotes have evolved signal transduction pathways called checkpoints to cope with genetic insults (2–4). Checkpoints stall progression through the cell cycle, providing time for cellular responses such as activation and re-localization of DNA repair enzymes to sites of DNA damage or transcriptional activation of specific genes. Checkpoint arrest can also lead to activation of apoptotic pathways perhaps under conditions when cell death is more beneficial to the organism as a whole than repair (reviewed in Refs. 5–7).

The *hRad9* gene was first identified based on sequence homology to the *rad9*$^+$ gene of the fission yeast *Schizosaccharomyces pombe* (8). In *S. pombe*, *rad9*$^+$ is required for the S-phase and G_2 checkpoints, which delay the onset of mitosis if DNA replication is incomplete or if DNA damage is present, respectively (3, 9–13). Five other *S. pombe* genes, *hus1*$^+$, *rad1*$^+$, *rad3*$^+$, *rad17*$^+$, and *rad26*$^+$ are also required for this response (10–13) and are also, with the exception of *rad26*$^+$, conserved in humans (14–17). Like their *S. pombe* orthologues, hRad9, hRad1, and hHus1, interact with each other in a stable complex (15, 18–20) that has recently been dubbed the 9-1-1 complex (21). Structural homology between each member of the 9-1-1 complex and PCNA has led to the hypothesis that the 9-1-1 complex replaces replication-associated PCNA-dependent functions during DNA repair (22–24). During DNA replication, the PCNA homotrimer forms a ring-like sliding clamp over DNA and acts to increase the processivity of DNA polymerase δ (25, 26). The 9-1-1/PCNA model is supported by the observation that hRad9, hRad1, and hHus1 each interact with hRad17 (27), which shares extensive homology to subunits of replication factor C, a protein required for loading PCNA[1] onto DNA (reviewed in Ref. 28). Furthermore, DNA damage induces not only the phosphorylation of hRad9 and hRad1 but also the association of 9-1-1 with chromatin (29). From this an attractive model has emerged in which hRad17-dependent loading of 9-1-1 onto DNA at sites of damage could co-ordinate the multifaceted checkpoint response.

Although these data suggest that hRad17 and 9-1-1 are early components of the checkpoint-signaling cascade, whether they are responsible for the initial detection of DNA damage still remains unclear. In *S. pombe*, the Rad3 protein, a phosphatidylinositol-related 3-kinase, phosphorylates Rad26 in response to DNA damage independently of the other checkpoint Rads (30), suggesting that it is the initiator of the checkpoint signaling cascade. Two human homologues of Rad3, ATM and ATR, phosphorylate a wide variety of cellular proteins on (S/T)Q sequences in response to DNA damage (31–38). Mutations in *ATM* result in the cancer predisposition syndrome, ataxia telangiectasia (39). Recently, hRad9 has been implicated as an ATM substrate (40). Although the evidence from fission yeast

* This work was supported by Canadian Institutes of Health Research Grants MOP-14352 and MOP-36526 and National Institutes of Health Grant E507940 (to S. D.). Flow cytometry was funded in part by Canadian Institutes of Health Research Grant MT-7827. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of a National Cancer Institute of Canada studentship.

** Recipient of U. S. Army Breast Cancer Research Studentship DAMD17-98-1-8080. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§§ A Cancer Care Ontario Scientist. To whom correspondence should be addressed: Cancer Research Laboratories, Queen's University, Kingston, Ontario, K7L 3N6, Canada. Tel.: 613-533-6923; Fax: 613-533-6830; E-mail: sd13@post.queensu.ca.

[1] The abbreviations used are: PCNA, proliferating cell nuclear antigen; DMEM, Dulbecco's modified Eagle's medium; RPE, retinal pigment epithelial; hTERT, human telomerase reverse transcriptase subunit; FBS, fetal bovine serum; PBS, phosphate-buffered saline; CIP, calf intestinal phosphatase; PAGE, polyacrylamide gel electrophoresis; IR, ionizing radiation.

TABLE I
Sequence of selected oligonucleotides used for hRad9 site-directed mutagenesis

Oligonucleotide	Sequence (5′ to 3′)
Selection Primer 1	GCTCTAGCCCTGGAGATGAAGTGC
Selection Primer 2	CAAGTAGCGGCCGGTAATTCCTGATTG
Selection Primer 3	GACAAGTAGCGGCAGGTAATTCCTGAT
T60A	ATACCAGGCAGCCGCCCCTGGTCAGGC
S160A	TGTTCTGCCCTTCGCTCCTGCACTGGCT
S277A	CAGGACCTGGGCGCCCCAGAGCGTCA
T292A	CAGGCTCACAGCGCACCCCACCCGGA
S328A	TCCATTTCCCTTGCACCTGGCCCCCA
S336G	AGCCCCCCAAGGGCCCCGGTCCCCA
T355A	CAGTGCCTGGGGCTCCCCCACCCAA
S375A	GCCCCTGTACGCGCCCCCCAGGGCCCC
S380G	CCCCAGGGCCCCGGCCCTGTGCTGGCG
S272A	CCGACTCGCACGCCCAGGACCTGGG

indicate that hRad9 is fulfilling a role in the G_2/M transition (11), ATM-dependent phosphorylation of hRad9 occurs regardless of cell cycle position and appears to be important for the G_1 DNA damage checkpoint (40). hRad9, through interactions with the anti-apoptotic Bcl-2 and Bcl-xL proteins, can also promote apoptosis and, therefore, appears to have a multifunctional role in responding to genotoxins (41).

Previous studies indicate that the hRad9 protein is extensively modified by phosphorylation under normal cellular conditions (19) and becomes hyper-phosphorylated in response to DNA damage at serine 272 (18, 40). Here, we further the current understanding of hRad9 phosphorylation by mapping sites required for its constitutive phosphorylation and by identifying of a novel, cell cycle-regulated, ionizing radiation-induced phosphorylation event.

EXPERIMENTAL PROCEDURES

Plasmids—The full-length *hRad9* cDNA was subcloned into unique *Xho*I and *Xba*I restriction sites of the pyDF31 mammalian expression vector. Protein expression from pyDF31 is driven by the strong constitutive SR α-promoter composed of the SV40 early promoter and a segment of the long terminal repeat of human T-cell leukemia virus (42). All *hRad9* point mutants were generated in pyDF31 using the transformer site-directed mutagenesis kit (CLONTECH, Palo Alto, CA) according to the manufacturer's instructions. Three selection primers were used to disrupt unique *Xho*I, *Not*I, and *Eag*I restriction sites in pyDF31-hRad9. The sequence of these primers and the *hRad9* mutagenic primers are shown in Table I. Constructs with multiple point mutations were made by sequential mutagenesis reactions or concurrently by using multiple mutagenic primers in the same reaction. The presence of the desired base substitutions were confirmed by DNA sequencing using an automated sequencer (Cortec DNA Services Laboratory, Kingston, ON and Canadian Molecular Research Services, Ottawa, ON, Canada).

Cell Culture and Transfections—HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C in 5% CO_2 atmosphere. The hTERT-RPE1 cell line, a human retinal pigment (RPE) cell line that stably expresses the human telomerase reverse transcriptase subunit (hTERT) (CLONTECH), was maintained as HeLa cells except in DMEM F12 media (Invitrogen). HeLa cells were transfected in 10-cm or 6-well plates using 20 μl or 2 μl of a 2:1 molar ratio of DOPE (1,2-dioleoyl-*sn*-glycerophosphatidylethanolamine) (Sigma) and DDAB (dimethyldioctadecylammonium bromide) (Sigma), respectively. The transfection reagent was mixed with 2 μg of DNA in 3.3 ml of DMEM (10-cm plate) or 0.25 μg in 600 μl of DMEM (6-well plate) and applied to cells for 4 h at 37 °C. The transfection solution was then replaced with DMEM plus 10% FBS, and cells were cultured for an additional 30–48 h before lysis.

Cell Synchronization and Flow Cytometry—HeLa cells cultured as described above were synchronized in early S-phase by double thymidine block as previously described (43). Cells were cultured to a confluence of ~30% and treated with 2 mM thymidine for 18 h. After 18 h, cells were released from thymidine for 8 h, treated for an additional 18 h, and then released for varying lengths of time. hTERT-RPE1 cells were synchronized in early S-phase using a single, 24-h dose of 5 mM thymidine. Mitotic HeLa cells were generated by treatment with 70 ng/ml demecolcine (Sigma). Synchronized cell populations were followed by flow cytometry; cells were harvested, resuspended in 1 ml of PBS and 1% FBS, fixed by the addition of 1 ml of 100% ethanol, and stored at 4 °C for at least 1 h. After fixing, cells were washed twice in PBS, resuspended in 1 ml of PBS, 1% FBS, and 0.5 mg/ml RNase A, and incubated for 40 min at 37 °C. Cells were then collected by centrifugation and resuspended in PBS, 50 μg/ml propidium iodide, and 0.1 mg/ml RNase A and analyzed using a flow cytometer (Beckman/Coulter EPICS Elite, Mississauga, ON).

Calf Intestinal Phosphatase (CIP) Treatment—Cells were lysed in 1 ml of NETN lysis buffer (250 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% Nonidet P-40) supplemented with 1% Triton-X, 20 μg/ml aprotinin, 4 μg/ml leupeptin, 2 mM sodium orthovanadate, 20 mM β-glycerophosphate, and 0.2 mM sodium fluoride. Lysates were incubated on ice for 30 min and centrifuged at 13000 × *g*. Supernatants were pre-cleared by the addition of 15 μl of α-chicken IgY-agarose (Promega, Madison WI) for 30 min at 4 °C before immunoprecipitation. The immunoprecipitation was performed with 1 μg of affinity-purified α-hRad9 polyclonal antibodies (Ref. 19; RCH antibodies, Kingston ON) and 15 μl of α-chicken IgY-agarose (Promega) for 2 h at 4 °C. Immunoprecipitated proteins were washed four times with 1 ml of cold PBS and resuspended in 50 μl of 0.2 × NE Buffer 3 (New England Biolabs, Mississauga, ON) and 1% SDS. Samples were boiled for 5 min and centrifuged at 6000 × *g* for 10 min. 20 μl of supernatant was incubated in the presence of 30 units of calf intestinal phosphatase (Roche Diagnostics) in 0.2 × NE Buffer 3 in a final volume of 200 μl for 30 min at 37 °C. The reaction was stopped by the addition of 100 μl of 3× SDS-PAGE sample buffer.

Metabolic Labeling—HeLa cells were transfected in a 6-well plate as described above. 24 h after transfection, the cells were washed 3× with phosphate-free DMEM (Life Technologies, Inc.) then incubated for 1 h in phosphate-free DMEM and 10% dialyzed FBS (dialyzed against 150 mM saline to deplete phosphate concentrations). Cells were labeled with 1.25 ml of phosphate-free DMEM containing 10% dialyzed FBS and 333 μCi of [^{32}P]orthophosphoric acid (PerkinElmer Life Sciences). Cells were further incubated at 37 °C in 5% CO_2 for 18 h. Cells were washed 3× with DMEM and 3× with PBS and harvested with a cell scraper. Cells were collected by microcentrifugation at 500 × *g* for 2 min. The supernatant was removed, and the cells were lysed in 400 μl of NETN buffer containing 0.4 mM 4-(2-aminoethyl)benzenesulfonyl fluoride HCl (AEBSF), 20 μg/ml aprotinin, 4 μg/ml leupeptin, 0.7 μg/ml pepstatin, 2 mM Na_3VO_4, 20 mM β-glycerophosphate, and 0.2 mM NaF. Lysates were incubated on ice for 1 h, then centrifuged at 16,000 × *g* for 20 min at 4 °C. Soluble cell lysates were immunoprecipitated with antibodies directed against hRad9 essentially as described above. Agarose was washed 4× with NETN buffer, resuspended in 60 μl of 2× electrophoresis buffer, and boiled for 5 min before SDS-PAGE (10% acrylamide). The gel was either transferred to nitrocellulose and immunoblotted with antibodies directed against hRad9 or dried using a gel slab dryer (Bio-Rad). Protein quantification of the immunoblot was performed using AlphaEase software and a Chemiimager (Alpha Innotech Corp., San Leandro, CA). ^{32}P-quantification was performed using ImageQuant software and a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunoblotting—All samples were boiled for 5 min before electrophoresis through 8 or 10% acrylamide, as indicated. Proteins were transferred to nitrocellulose (Amersham Pharmacia Biotech) using a semi-dry transfer cell (Bio-Rad) or a wet transfer apparatus (Hoefer Scientific, San Francisco, CA). Membranes were blocked for 1 h in 5% nonfat milk powder in PBS plus 0.1% Tween 20 and then incubated in affinity-purified α-hRad9 polyclonal chicken antibodies at 50 ng/ml in PBS for 1–18 h at 4 °C. After extensive washing in PBS plus 0.1% Tween 20, blots were incubated in horseradish peroxidase-conjugated α-chicken secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a final dilution of 1 in 50,000 for 45 min at 4 °C. Membranes washed and incubated in Western blot chemiluminescence reagent plus (PerkinElmer Life Sciences) before exposure to x-ray film (Eastman Kodak Co.).

RESULTS

hRAD9 Is Constitutively Phosphorylated on Serine/Threonine-Proline Sequences—The hRad9 protein is constitutively phosphorylated in the absence of DNA damage (19) and becomes additionally phosphorylated when DNA damage is present (18). We have observed that under normal cellular conditions (*i.e.* those where cells are not exposed to exogenous stresses) overexpressed hRad9 consists of four species that

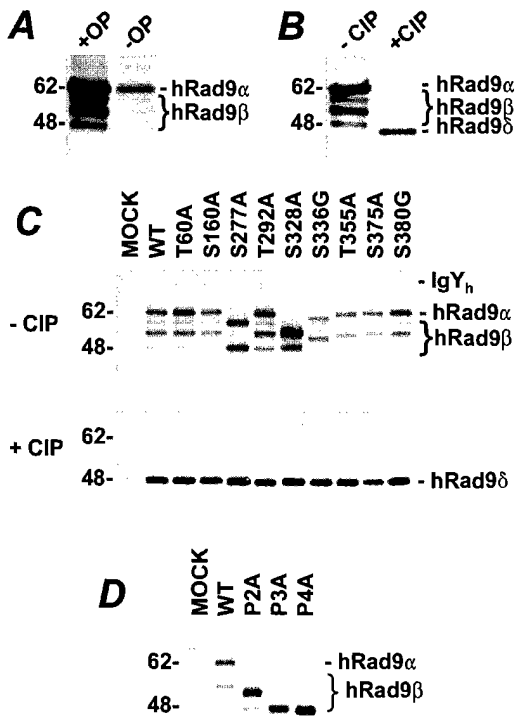


FIG. 1. **hRad9 is constitutively phosphorylated on (S/T)P sequences.** *A*, exogenous, overexpressed hRad9 from transiently transfected HeLa cells (+*OP*) or endogenous hRad9 from untransfected HeLa cells (−*OP*) was immunoprecipitated with polyclonal antibodies directed against hRad9. Immunoprecipitated proteins were subjected to SDS-PAGE (8%) and immunoblotted with antibodies directed against hRad9. *B*, hRad9 overexpressed in HeLa cells was immunoprecipitated with antibodies directed against hRad9 and then incubated in the presence (+*CIP*) or absence (−*CIP*) of calf intestinal phosphatase prior to SDS-PAGE (8%) and immunoblotting. The +CIP sample was diluted before electrophoresis for presentation purposes. *C*, each of the nine serine and threonine amino acids that are followed immediately by proline in the hRad9 amino acid sequence were converted to non-phosphorylatable alanine or glycine residues (as indicated) by site-directed mutagenesis. Plasmids encoding wild type (*WT*) hRad9 and each mutant protein were used to transfect HeLa cells. Cellular proteins were immunoprecipitated and incubated in the presence (*lower panel*) or absence of CIP (*upper panel*). Proteins were then separated by SDS-PAGE (8%) and immunoblotted with antibodies directed against hRad9. *D*, the four mutations that caused mobility shifts in *A* were introduced to the hRad9 cDNA sequentially in combination. Wild type, P2A (S328A + S336G), P3A (P2A + S277A), and P4A (P3A + T355A) proteins were expressed in HeLa cells and immunoblotted as before.

differ in migration rate on SDS-PAGE as visualized by immunoblot analysis. The slowest migrating of these species, which we have termed hRad9α, co-migrates with the majority of endogenous hRad9 at an apparent molecular mass of ~60 kDa (Fig. 1*A*). *In vitro* dephosphorylation of exogenous hRad9 causes each of its four migratory forms to collapse into a single band at about 45 kDa, which we have called hRad9δ (Fig. 1*B*). These data suggest that relative to endogenous hRad9, a large portion of the overexpressed protein is only partially phosphorylated and, hence, has various migratory forms. We have collectively designated all of these partially phosphorylated forms hRad9β. Previous work in our lab has shown that deleting the C terminus of exogenous hRad9 can reduce the number of differentially migrating species from four to one,[2] indicating that the C terminus of hRad9 is required for constitutive phosphorylation of the protein.

With this in mind, we used site-directed mutagenesis to identify amino acid residues required for the constitutive phosphorylation of hRad9. Potential phosphorylated residues near the C terminus of the protein were converted to non-phosphorylatable amino acids. Mutants were expressed in HeLa cells after transient transfection and screened for migratory shifts as detected by Western analysis. The gross overexpression of protein by the strong SR α-promoter allowed us to distinguish plasmid-derived hRad9 protein from the endogenous protein simply by limiting the exposure time to x-ray film. A trend between those mutants with altered Western blot banding patterns soon emerged, as each of these mutants contained disrupted serine or threonine residues followed immediately by a proline (results summarized in Table II). The (S/T)P motif is the minimum consensus sequence for the cyclin-dependent kinase family of kinases (44). We therefore mutated all nine (S/T)P sequences in hRad9, immunoprecipitated these proteins from transfected HeLa cells, and compared their banding pattern to the wild type protein (Fig. 1*C*). Of the nine (S/T)P mutants, four exhibit changes in hRad9 mobility on SDS-PAGE, suggesting that they are sites of phosphorylation. The S277A and S328A mutants exhibited complete loss of two bands each. The S336G mutation caused an increase in mobility of three of the four bands, whereas the T355A mutation caused a slightly increased mobility in all four of the hRad9α and β forms (Fig. 1*C*; *upper panel*). When dephosphorylated by treatment with calf intestinal alkaline phosphatase, each protein migrated at about 45 kDa (hRad9δ), indicating that the mobility shifts in the upper panel are indeed the result of phosphorylation changes (Fig. 1*C*; *lower panel*). Sequentially introducing these four mutations in combination leads to a reduction in the number of mobility forms and a progressive increase in the mobility of hRad9 (Fig. 1*D*). Although the P2A protein (S328A + S336G) migrated as a doublet, P3A (P2A + S277A) migrated as a single band with slightly less mobility than P4A (P3A + T355A). This subtle shift resulting from the T355A mutation is consistent with that observed in Fig. 1*C* as well as Figs. 6, *A* (*panel 2*) and *B*. P4A migrated with an approximate molecular mass of 45 kDa (Fig. 1*D*), which is only slightly larger than the predicted molecular mass of 42.5 kDa for hRad9.

hRad9 Is Constitutively Phosphorylated on Sites Other than (S/T)P Sequences—The P4A mutant still exhibited a modest mobility shift when treated with alkaline phosphatase (Fig. 2*A*). This suggested the existence of additional sites of constitutive phosphorylation in hRad9. To determine whether these

TABLE II
Summary of characterized hRad9 phosphorylation mutants transiently expressed in HeLa cells

Amino acid	Context[a]	Immunoblot shift[b]
Serine 380	GP**S**PVL	No
Serine 375	VR**S**PQG	No
Serine 368	FG**S**ILA	No
Serine 363	FR**S**LEF	No
Threonine 355	PG**T**PPP	Yes
Threonine 351	PS**T**VPG	No
Serine 350	EP**S**TVP	No
Serine 341	PH**S**EEE	No
Serine 336	PK**S**PGP	Yes
Serine 328	SL**S**PGP	Yes
Tyrosine 306	DS**Y**MIA	No
Threonine 292	HS**T**PHP	No
Serine 277	LG**S**PER	Yes
Serine 272	SH**S**QDL	Yes (IR-induced)
Serine 160	PF**S**PAL	No
Threonine 60	AA**T**PGQ	No

[a] Amino acids surrounding the residue of interest (indicated in boldface).
[b] Immunoblot mobility shift resulting from phosphorylation at the residue of interest.

[2] R. P. St.Onge and S. Davey, unpublished results.

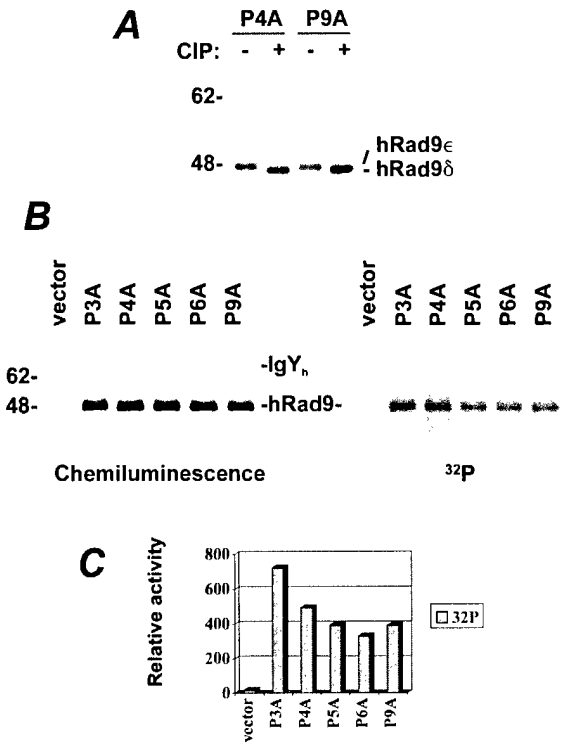


FIG. 2. **hRad9 is constitutively phosphorylated at sites other than (S/T)P sequences.** *A*, P4A and P9A forms of hRad9 were expressed in HeLa cells by transient transfection and immunoprecipitated with antibodies directed against hRad9. Immunoprecipitated proteins were left untreated (−) or treated with CIP(+), size-fractionated by SDS-PAGE (8%), and immunoblotted with antibodies directed against hRad9. *B*, hRad9 protein was immunoprecipitated from P3A, P4A, P5A, P6A, P9A, and empty vector-transfected HeLa cells that were metabolically labeled with inorganic ^{32}P for 18 h before lysis. A portion of each immunoprecipitated, radiolabeled protein was size-fractionated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies against hRad9 (*left panel*). A second, identical gel was dried and exposed to a PhosphorImager screen for 3 days (*right panel*). *C*, quantification of the ^{32}P signal from the *right panel* of Fig. 2*B* as determined by the ImageQuant software program. Values were normalized ^{32}P background in each lane and to the chemiluminescence signal from the *left panel* of Fig. 2*B*.

additional sites were (S/T)P sites whose phosphorylation produced little or no shift on a Western blot, we mutated all nine (S/T)P sites in hRad9 in combination. The migration of the P9A mutant (P4A and T60A, S160A, T292A, S375A, and S380G) was not readily distinguishable from that of the P4A mutant and was still sensitive to phosphatase treatment (Fig. 2*A*). Therefore, despite being constitutively phosphorylated on at least four (S/T)P sites, hRad9 is also phosphorylated on sites other than these sequences. We have called the form of hRad9, which completely lacks phosphorylation at (S/T)P sites but still remains phosphorylated, hRad9ε.

To further characterize the constitutive phosphorylation of hRad9, HeLa cells were transfected with a series of hRad9 mutants or empty vector and metabolically labeled with [^{32}P]orthophosphoric acid. The hRad9 point mutants, P3A, P4A, P5A (P4A + T292A), P6A (P5A + S375A), and P9A were used in the transfection, and after metabolic labeling, the hRad9 protein was immunoprecipitated from these cells. Immunoprecipitated protein was size-fractionated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with antibodies directed against hRad9 (Fig. 2*B*; *left panel*). The amount of hRad9 protein recovered from each immunoprecipitation was defined by densitometric analysis of the immunoblot using a Chemiimager and was used for data normalization as described below. A second, identical gel was also run, dried, exposed to a phosphor screen, and analyzed using a STORM PhosphorImager (Fig. 2*B*; *right panel*). In agreement with Fig. 2*A*, each hRad9 mutant, including P9A, was significantly phosphorylated, confirming that the hRad9 protein is constitutively phosphorylated at sites other than (S/T)P sites.

We went on to quantitate the ^{32}P signal in Fig. 2*B* using the ImageQuant software program (Fig. 2*C*). Values were normalized to background ^{32}P in each lane and to protein levels (which were all within 15%) determined above by spot densitometry of the hRad9 immunoblot. Because only the P3A mutant was wild type at threonine 355, a site we knew to be constitutively phosphorylated (see Fig. 1), we expected that P3A should have a stronger signal than each of the other four mutants, and it did. The remaining mutants exhibited smaller differences in relative ^{32}P activity, which may be a reflection of confounding variables inherent to the experiment, such as protein normalization, rather than legitimate differences in phosphorylation.

hRad9 Phosphorylation on Serine 272 in Response to Ionizing Radiation Does Not Require Constitutive Phosphorylation at (S/T)P sites—ATM and ATR, two serine/threonine DNA damage-responsive kinases that likely function in the same biochemical pathway as hRad9, have been demonstrated to phosphorylate target proteins at serine/threonine-glutamine ((S/T)Q) sequences (33). Because serine 272 of hRad9 is followed immediately by a glutamine residue, we hypothesized that this amino acid was the site of the previously reported ionizing radiation (IR)-induced phosphorylation of hRad9 (18). In this regard, we observed that a serine to alanine mutation at serine 272 had no effect on constitutive phosphorylation but abrogated the ability of exogenously expressed hRad9 to become phosphorylated in response to ionizing radiation (Fig. 3*A*). Although sub-populations of both the α and β forms of wild type hRad9 (*WT*) underwent a subtle mobility change when cells were treated with 20 and 40 gray doses of IR (hRad9α to hRad9γ and hRad9β to hRad9β(γ)), the S272A mutant showed no changes in hRad9α or hRad9β mobility (Fig. 3*A*; *upper panel*). This shift was confirmed to be the result of phosphorylation, because CIP treatment of these samples yielded co-migrating dephosphorylated proteins (hRad9δ, Fig. 3*A*; *lower panel*). Although the subtle nature of the mobility shift combined with the complex banding pattern of hRad9 has made this effect difficult to observe by immunoblotting techniques, we have found this result to be reproducible and offer further evidence in support of it in Figs. 3, *B* and *C*. This observation also confirms a recent report that demonstrated that ionizing radiation induced phosphorylation at this residue and that this phosphorylation was ATM-dependent (40). This report, however, like all previous studies demonstrating IR-induced phosphorylation of hRad9, involved a constitutively phosphorylated protein (18,29). Although the purpose of this constitutive phosphorylation remains unknown, it may be potentiating some aspect of hRad9 cellular activity. The observation in Fig. 3*A* that the β forms of wild type hRad9, like the α form, shift subtly in response to IR, seems to indicate that (S/T)P phosphorylation of hRad9 is not required for IR-induced phosphorylation at serine 272.

To address this directly, we tested the response to IR of hRad9 P9A, which lacks all phosphorylatable (S/T)P sites. The P9A hRad9 mutant was expressed in cells that were subsequently irradiated or mock-irradiated. P9A was immunoprecipitated from these cells 1 h later, treated with phosphatase as indicated, size-fractionated by SDS-PAGE, and immunoblotted with antibodies directed against hRad9 (Fig. 3*B*). In response to irradiation, a slower migrating form of hRad9ε (hRad9ε(γ)) became readily apparent. Both hRad9ε and hRad9ε(γ) increased in mobility and co-migrated when treated with CIP

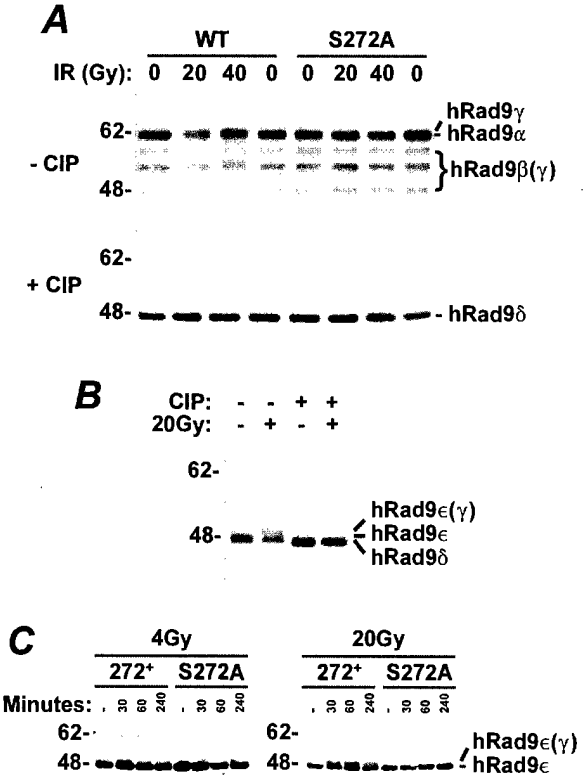


FIG. 3. **Ionizing radiation-induced phosphorylation at serine 272 of hRad9 does not require constitutive phosphorylation at (S/T)P sequences.** *A*, wild type (*WT*) hRad9 and S272A, harboring a serine to alanine mutation at the putative ATM phosphorylation site, were expressed in HeLa cells that were exposed 0, 20, and 40 gray of ionizing radiation, as indicated. One hour later cell lysates were immunoprecipitated and left untreated (*upper panel*) or treated with CIP (*lower panel*) before SDS-PAGE (8%) and immunoblotting with antibodies directed against hRad9. *B*, the P9A protein was immunoprecipitated from cells 1 h after irradiation with 20 gray, as indicated above. Immunoprecipitated hRad9 was then treated with CIP as indicated, size-fractionated by SDS-PAGE (8%), and immunoblotted as before. *C*, the S272A mutation was introduced into the mutant described previously. P9A and P9A + S272A were expressed in HeLa cells, treated with 0 (−), 4 (*left panel* only) or 20 gray (*right panel* only) of ionizing radiation and harvested at 30, 60, or 240 min later, as indicated. Lysates were immunoblotted with antibodies directed against hRad9.

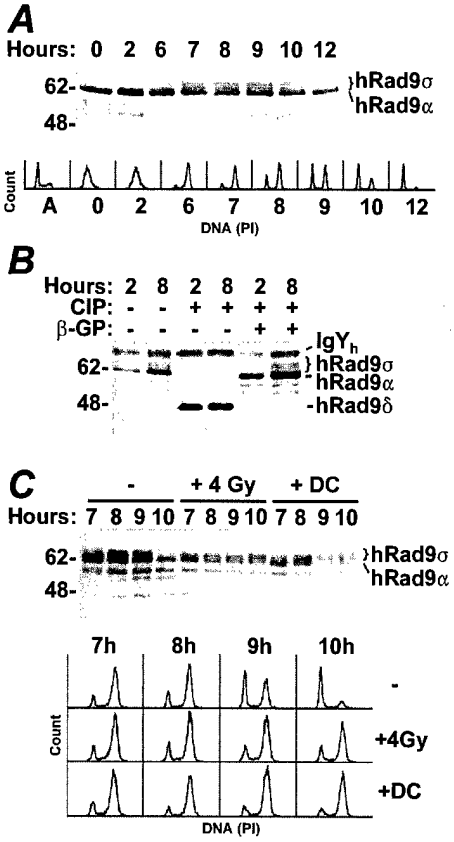


FIG. 4. **Constitutively phosphorylated hRad9 is hyper-phosphorylated in G_2 and M phases of HeLa cells.** HeLa cells were synchronized in early S-phase using a double thymidine block, released, and harvested 0, 2, 6, 7, 8, 9, 10, and 12 h later. Cells from each time point were either lysed, size-fractionated by SDS-PAGE (10%), and immunoblotted with antibodies against hRad9 (*top*) or stained with propidium iodide to measure DNA content before analysis by flow cytometry (*bottom*). *B*, S-phase and G_2/M HeLa cells, harvested 2 and 8 h after release from a double thymidine block respectively, were lysed, and the hRad9 was immunoprecipitated. hRad9 protein from each time point was then treated with CIP in the presence or absence of the phosphatase inhibitor β-glycerophosphate (*β-GP*). Proteins were separated by SDS-PAGE (10%) and immunoblotted as above. *C*, late S-phase HeLa cells, generated from a single thymidine block and release, were left untreated (−), treated with 4 gray of IR to delay cells in G_2 (*+4 Gy*) or 1.7 μg/ml microtubule inhibitor demecolcine (*+DC*) to arrest cells in mitosis. Cells were harvested 7, 8, 9, and 10 h after thymidine release (1.5, 2.5, 3.5, and 4.5 h after IR and demecolcine administration) and used for immunoblotting (*top*) and flow cytometry (*bottom*) as in *A*. *PI*, propidium iodide.

(hRad9δ), indicating that hRad9ϵ(γ) was in fact a phosphorylated form of hRad9ϵ. To confirm that the IR-induced phosphorylation of hRad9ϵ was occurring at serine 272, the S272A mutation was introduced into the P9A mutant, and these proteins were analyzed for mobility changes after exposure to low and high doses of IR (Fig. 3*C*). Consistent with Fig. 3*B*, the P9A mutant migrated as a doublet at all time points after 20 gray of irradiation, although the slower migrating species was less abundant at 240 min post-IR. The formation of this doublet was not observed, however, when the S272A mutation was introduced into the P9A mutant background (Fig. 3*C*; *far right*). At the lower dose of 4 gray (Fig. 3*C*; *left panels*) this mobility shift was much less pronounced and did not persist as long, indicating a dose-dependent response typical of checkpoint control. Collectively, these results indicate that constitutive phosphorylation at (S/T)P sites is not essential for the IR-induced phosphorylation of hRad9 at serine 272.

hRad9 Is Phosphorylated in a Cell Cycle-dependent Manner in HeLa Cells—Given that hRad9 is phosphorylated on potential cyclin-dependent kinase consensus sites, we hypothesized that the attachment of these seemingly constitutive phosphate groups may be regulated in a cell cycle-dependent manner. Therefore, a double thymidine block was used to generate synchronized cell populations, which were examined for differences in endogenous hRad9 phosphorylation by immunoblotting (Fig. 4*A*; *top*). Cell cycle position was monitored by flow cytometry of propidium iodide-stained nuclei (Fig. 4*A*; *bottom*). Consistent with a recent report (40), hRad9α, which we have concluded to be highly phosphorylated at C-terminal (S/T)P sites, was observed in all phases of the cell cycle (Fig. 4*A*). However, even slower migrating forms of hRad9, migrating at an apparent molecular mass of ~65 kDa (hRad9σ), appeared in samples collected during G_2/M (Fig. 4*A*; *samples 7, 8, 9,* and *10*). We went on to demonstrate that this mobility shift was the result of a cell cycle-specific phosphorylation event because the hRad9α and hRad9σ bands in G_2/M HeLa cells collapsed into a single faster-migrating hRad9δ band at 45 kDa (Fig. 4*B*).

To address whether this phosphorylation change was occurring in G_2, mitosis, or both, late S-phase HeLa cells were either irradiated with 4 gray of IR to delay cells in G_2, treated with the microtubule-inhibiting drug demecolcine to arrest cells in mitosis, or left untreated and allowed to cycle into G_1. Consist-

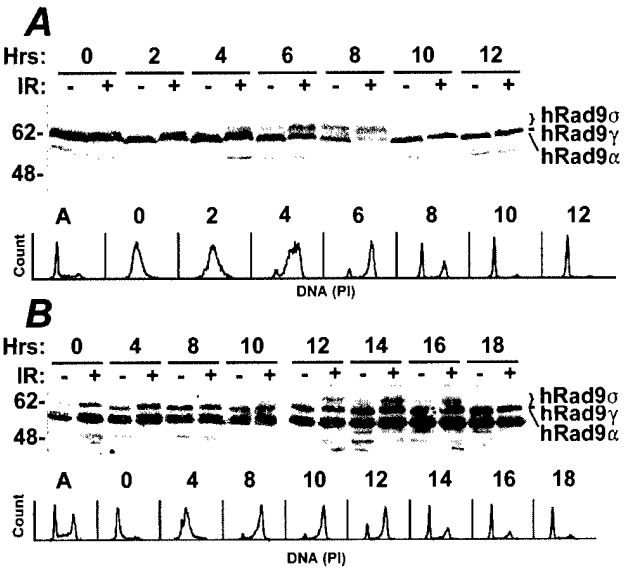


FIG. 5. **Phosphorylation of hRad9 in G_2/M is enhanced by IR but is distinct from phosphorylation at serine 272.** *A*, HeLa cells, synchronized with a double thymidine block and release, were analyzed by immunoblotting with antibodies directed against hRad9. At each time point, cells were either left as is or irradiated with 20 gray 30 min before harvest, as indicated. Cells from each time point were also followed by flow cytometry (*bottom*). *B*, similar to *A*, only using hTERT-RPE1 cells that were synchronized with a single thymidine block and release. *PI*, propidium iodide.

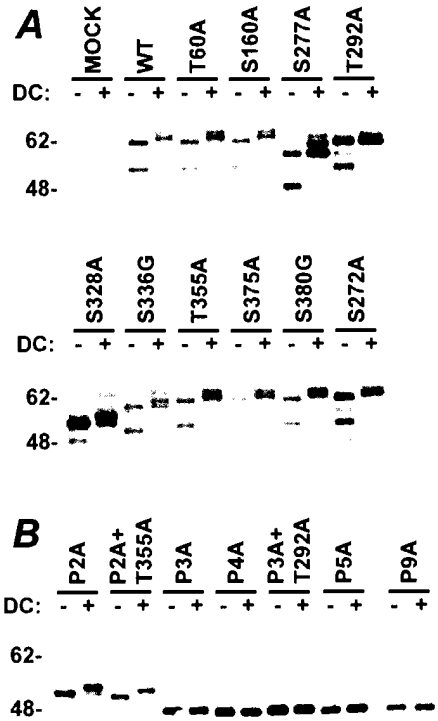


FIG. 6. **Cell cycle-regulated phosphorylation of hRad9 is dependent on threonine 292.** *A*, HeLa cells, mock or transiently transfected with each of the single (S/T)P hRad9 mutants or the S272A ATM consensus site mutant, were either left untreated or treated with demecolcine for 18 h before harvest. Cells were subjected to SDS-PAGE (8%) followed by immunoblotting for hRad9 (*upper*) or flow cytometry analysis to confirm mitotic arrest (*lower*). *B*, similar to *A*, only using mutants with multiple (S/T)P disruptions. (P2A = S328A + S336G; P3A = P2A + S277A; P4A = P3A + T355A; P5A = P4A + T292A; P9A = P5A + T60A, S160A, S375A, and S380G). *DC*, demecolcine.

ent with Fig. 4*A*, in the untreated cells, the hyper-phosphorylated hRad9σ form(s) was present in G_2 and M but not when cells had cycled into G_1 (Fig. 4*C*; first 4 *lanes*, *7–10 h*). If cells were treated with either a low dose of IR or demecolcine, cells did not progress into G_1, and hRad9σ persisted (Fig. 4*C*; 10-h time points). Taken together, these results indicate that in HeLa cells, endogenous hRad9 is hyper-phosphorylated in G_2 and mitosis on an amino acid residue(s) distinct from the constitutive phosphorylation sites we have identified.

Cell Cycle-dependent Phosphorylation of hRad9 Is Enhanced by IR but Is Distinct and Independent of Phosphorylation at Serine 272 of hRad9—To confirm that the cell cycle-regulated hyper-phosphorylation of hRad9 was distinct from that induced by high doses of ionizing radiation at serine 272, synchronized cells were irradiated with 20 gray of IR at various stages of the cell cycle 30 min before their harvest. Consistent with Fig. 3*A* and the findings of Chen *et al.* (40), in Fig. 5*A* it was observed that endogenous hRad9 was shifted in a subtle manner in response to ionizing radiation regardless of cell cycle position (hRad9γ). The appearance of hRad9γ occurred independently and was distinct from hRad9σ in G_2/M cells (Fig. 5*A*; 6- and 8-h time points). Furthermore, the amount of hRad9σ was modestly increased if cells were treated with ionizing radiation before harvest at time points 4, 6, 8, and to a lesser extent 10, when most cells were in G_1.

To address whether the cell cycle-regulated phosphorylation of hRad9 was cell type-specific, occurring exclusively in HeLa cells, we repeated this experiment in hTERT-RPE1 cells (Fig. 5*B*). These cells have an indefinite life span due to stable expression of the human telomerase reverse transcriptase subunit but maintain the normal karyotype of primary epithelial cells. As in Fig. 5*A*, hRad9 in the hTERT-RPE1 cells underwent a modest mobility shift in response to IR (hRad9γ) at all stages of the cell cycle (Fig. 5*B*). hRad9σ in G_2/M hTERT-RPE1 cells, however, was much less prevalent in the absence of IR compared with that observed for the HeLa cell line. Nevertheless, upon irradiation the abundance of hRad9σ increased significantly (Fig. 5*B*; *12*, *14*, and *16 h*), indicating that hRad9 undergoes cell cycle-dependent IR-induced phosphorylation in both HeLa cells and hTERT-RPE1 cells.

Efficient Cell Cycle-regulated Hyper-phosphorylation of hRad9 Requires Serine 277 and Threonine 292—To address whether the cell cycle-regulated hyper-phosphorylation of hRad9 was dependent on phosphorylation at (S/T)P sites, we expressed each of the nine single (S/T)P mutants in HeLa cells and examined these mutants for the cell cycle-dependent mobility changes we observed in the endogenous protein (Figs. 4 and 5). The S272A ATM consensus site mutant was also included in this experiment. Transfected HeLa cells were arrested in mitosis by treatment with demecolcine, lysed, and then immunoblotted with antibodies directed against hRad9 (Fig. 6*A*). The absence of signal in the mock-transfected lane confirmed that any mobility changes were derived from the overexpressed protein and not endogenous hRad9. Propidium iodide staining was used to confirm the effectiveness of the demecolcine treatment. In each of the transfected cell populations, demecolcine treatment resulted in an increase in the number of cells in G_2/M from 20–29% in the untreated, asynchronous cells, to 67–82% in the demecolcine-treated cells.[2] When cells were arrested in mitosis, all proteins exhibited a significant reduction in the abundance of the faster-migrating hRad9β forms. Furthermore, the majority of the wild type protein existed as two species that migrated slower through SDS-PAGE than the hRad9α form from asynchronous cells (Fig. 6*A*; *upper panel*). These species are similar to the hRad9σ forms observed in the G_2/M samples of Fig. 4*A*. The cell cycle-dependent hyper-phosphorylation of hRad9 was also observed in the S272A mutant protein (Fig. 6*A*; *lower panel*) as well as

each of the single (S/T)P mutants with two notable exceptions. When the constitutively phosphorylated serine 277 is mutated to alanine, the efficiency of this mitotic hyper-phosphorylation is drastically reduced because most of the protein co-migrates with the slowest-migrating S277A form in the untreated cells. Mutational inactivation of threonine 292 results in the significant reduction of the fastest-migrating hyper-phosphorylated species and the complete loss of the slowest migrating hRad9σ form (Fig. 6*A*; *upper panel*).

These observations were confirmed using hRad9 mutant proteins harboring multiple mutations at (S/T)P sites (Fig. 6*B*). When multiple constitutively phosphorylated (S/T)P sites were disrupted but serine 277 was left intact (P2A and P2A + T355A), the protein was still efficiently hyper-phosphorylated when cells were mitotic. Upon introduction of the S277A mutation in these proteins (P3A and P4A), the efficiency of this hyper-phosphorylation was drastically reduced, just as it was for the S277A single mutant. When the T292A mutation was introduced into the P3A and P4A proteins (P3A + T292A and P5A), the hyper-phosphorylation was no longer apparent. Not surprisingly, the same was observed for the P9A mutant, which lacks all nine hRad9 (S/T)P sites.

DISCUSSION

We have mapped four amino acids that are constitutively phosphorylated in the hRad9 checkpoint protein. These amino acids (serine 277, serine 328, serine 336, and threonine 355), when converted to non-phosphorylatable residues, alter the mobility of hRad9 in SDS-PAGE in a way that is consistent with decreased phosphorylation (Fig. 1). Each of the four phosphorylated residues we have identified is followed immediately by proline in the primary amino acid sequence of hRad9. Based on this observation, the kinase(s) phosphorylating these sites in hRad9 could belong to the cyclin-dependent family of kinases. Several cyclin-dependent kinases recognize the (S/T)P motif as a minimal consensus site in target substrates. The preferred cyclin-dependent kinase consensus site of (S/T)PX(K/R) is only represented at serine 277 of hRad9 (S^{277}PER). A proline at position four, which some evidence indicates may be tolerated by cyclin-dependent kinases (45), is found in each of the remaining three constitutive hRad9 sites we have identified (S^{328}PGP, S^{336}PGP, and T^{355}PPP). Threonine 292, which we conclude to be required for the cell cycle-dependent phosphorylation of hRad9 represents the only other hRad9 (S/T)P site with a proline at position four (T^{292}PHP). The four remaining (S/T)P sequences in the protein (threonine 60, serine 160, serine 375, and serine 380) contain neither P, R, nor K at position 4 (Table II). This in addition to our observations that mutating these residues does not alter the mobility of hRad9 through SDS-PAGE (Figs. 1*C*, 2*A*, 6, *A* and *B*) or significantly reduce ^{32}P uptake in metabolically labeled cells (Fig. 2*C*), suggests that these four sites are not constitutively phosphorylated.

Although we have termed the (S/T)P phosphorylation sites constitutive, we cannot rule out the possibility that phosphorylation at these sites is regulated in a complex manner. Several groups using different cell lines and antibodies have observed as we have that endogenous hRad9 exists primarily as a single species migrating at 60 kDa on a Western blot (hRad9α). However, we have occasionally observed bands in the 45–60-kDa range when studying endogenous hRad9 that correlate with the partially phosphorylated bands of the overexpressed protein (hRad9β). Even though we are able to limit the abundance of these bands by increasing the concentration of phosphatase inhibitors in our lysis buffer or by lysing cells directly in SDS-PAGE sample buffer, we cannot rule out the possibility that constitutively phosphorylated hRad9 intermediates are physiologically significant. Importantly, however, no consistent changes were observed in the 45–60-kDa bands at different stages of the cell cycle (Figs. 4*A* and 5, *A* and *B*), suggesting that phosphorylation at serine 277, serine 328, serine 336, and threonine 355 remains constant throughout the cell cycle.

Recently, a phospho-specific antibody directed against the serine 272 of hRad9 was used to demonstrate that IR-induced phosphorylation of hRad9 occurs at this site *in vivo* (40). We have confirmed these findings using an independent method by showing that a serine to alanine mutation at residue 272 abrogates hRad9 phosphorylation when asynchronous cells are given a high dose of IR (Fig. 3*A*). The observation made by Chen *et al.* (40) that phosphorylation at this residue is dependent on ATM raises many interesting questions regarding the detection of IR-induced DNA damage and the initiation of the checkpoint response in general. In further agreement with the findings of Chen *et al.* (40) is our observation that phosphorylation at serine 272 occurs independently of cell cycle position (Figs. 3*A* and 5, *A* and *B*). Two lines of evidence indicate that this occurs independently of (S/T)P phosphorylation as well. First, when cells are treated with high doses of IR, subtle migratory changes in both the α and β forms of overexpressed hRad9 are observed that are not observed in a S272A mutant (Fig. 3*A*). Second, mutational inactivation of the (S/T)P phosphorylation sites still yields a protein (hRad9ϵ) that is capable of serine 272 phosphorylation in response to IR (Fig. 3, *B* and *C*).

We also report the identification a second hyper-phosphorylation event for hRad9 that is cell cycle-regulated. We first identified these phospho-forms (hRad9σ) in HeLa cells that had been synchronized in G_2 or mitosis and found that we could moderately increase their abundance by treating these cells with ionizing radiation. In contrast, we found that hTERT-RPE1 cells, a karyotypically normal human epithelial cell line, displayed practically no hRad9σ in G_2 and M unless cells were irradiated at this cell cycle position. The most plausible explanation for this inconsistency is that even in the absence of IR, the highly proliferative, cancerous HeLa cells contain sufficient endogenous DNA damage to trigger the checkpoint response. Hence, in the absence of IR this cell cycle-regulated and DNA damage-responsive phosphorylation event is readily detectable in HeLa cells. Based on our mutagenesis studies, we can conclude that the cell cycle-regulated phosphorylation of hRad9 occurs at a site(s) other than those we have identified as constitutive phosphorylation sites (serine 277, serine 328, serine 336, or threonine 355) and does not occur on the IR-induced phosphorylation site at serine 272.

Although phosphorylation at serine 272 is independent of cell cycle position and (S/T)P phosphorylation, the cell cycle-regulated phosphorylation of hRad9 is dependent on both cell cycle position and constitutive (S/T)P phosphorylation. Specifically, a S277A mutation reduces not only the constitutive phosphorylation of hRad9 but also its ability to undergo cell cycle-regulated hyper-phosphorylation when cells are arrested in mitosis (Fig. 6). Each of the other constitutive sites we have identified does not exhibit this behavior because mutational inactivation of serine 328, serine 336, and threonine 355 singly or in combination still yields a protein capable of efficient cell cycle-regulated hyper-phosphorylation (Fig. 6). Substituting threonine 292 with non-phosphorylatable alanine, however, completely abrogates one of the G_2/M hyper-phosphorylated species of hRad9 and reduces the abundance of the other (Fig. 6). Interestingly, threonine 292 is the only (S/T)P site that is conserved between hRad9 and *S. pombe* Rad9 (46). The complete loss of one of the hRad9σ forms in a T292A mutant may

indicate that threonine 292 is being phosphorylated in G_2/M. An alternative explanation is that threonine 292 is constitutively phosphorylated and, like serine 277, is a prerequisite for hyper-phosphorylation of hRad9. Although the T292A mutation yields no detectable electrophoretic mobility change under normal conditions (Figs. 1*C*, 2*A*, 6, *A* and *B*), a modest decrease in ^{32}P uptake was observed in ^{32}P-labeled cells (Fig. 2*C*; P4A compared with P5A, P6A, and P9A). Therefore, although the cell cycle-regulated hyper-phosphorylation is clearly dependent on threonine 292, further experimentation will be required to fully resolve the nature of this dependence.

Perhaps of further interest is the observation that the hRad9β forms, whose abundance normally exceed that of the hRad9α form when the protein is overexpressed (see Fig. 1), are all but absent during a demecolcine-induced mitotic arrest (Fig. 6*A*). This could be the result of destabilization or further phosphorylation of hRad9β at the G_2/M transition. Whether this has any relevance to the endogenous protein, however, which exists predominantly in the hRad9α form, remains to be seen.

There has been much speculation recently that the association of hRad9 with hRad1 and hHus1 results in the formation of a ring-like heterotrimer that encircles the DNA double helix. Although the crystal structure of this complex has yet to be solved, structural predictions using the primary amino acid sequence of these three proteins indicate similarity to the PCNA homotrimer, a ring-like complex that acts as a sliding clamp over DNA. In these modeling studies, the entire length of hRad1 and hHus1 are used, but only the first 280 amino acids of hRad9 fit the predicted PCNA-like model (24). The SQ and (S/T)P sites critical for hRad9 phosphorylation sites we have reported here are located at either the very end of this region (serine 272 and serine 277) or C-terminal to it (threonine 292, serine 328, serine 336, and threonine 355). Because some data suggest that phosphorylation at these residues may be important for 9-1-1 assembly (18, 21), the C terminus of hRad9 may be acting as a regulatory domain for assembly of this complex. Alternatively, the attachment of these phosphates could influence some other aspect of hRad9 function, such as its pro-apoptotic role.

Although we have now identified two distinct forms of damage-dependent hRad9 phosphorylation, mapped at least four sites of constitutive phosphorylation, and characterized the interdependence of these events, many questions remain regarding the nature and function of hRad9 phosphorylation. These include the location of the remaining constitutive sites of phosphorylation and the identification of proteins responsible for the addition and removal of phosphates. In addition, it remains unclear whether the cell cycle-regulated phosphorylation of hRad9 occurs in G_2 and persists through mitosis or whether these are two separate events. In any event, this is the first evidence linking the hRad9 protein to the G_2/M transition, a transition in which the *S. pombe* Rad9 protein plays an instrumental regulatory role. Furthermore, the discovery that hRad9 undergoes at least two distinct phosphorylation events in response to IR raises other interesting questions. It is known that hRad9 hyper-phosphorylation in response to IR occurs concurrently with its association with chromatin (29), and although it has yet to be shown directly, there is likely an interdependence between these two events. Which IR-induced phosphorylation of hRad9 coincides with its association with DNA is currently an unresolved issue.

Acknowledgments—We thank Dr. David LeBrun and Dr. Susan Cole for helpful discussion. We also thank Kathy Kennedy, Derek Schulze, and Maureen Rogers for technical assistance and Marie Evangelista, Lee Fraser, and Deborah Greer for critically reading the manuscript.

REFERENCES

1. Loeb, L. A. (1991) *Cancer Res.* **51,** 3075–3079
2. Hartwell, L. H., and Weinert, T. A. (1989) *Science* **246,** 629–634
3. Weinert, T. A., and Hartwell, L. H. (1988) *Science* **241,** 317–322
4. Hartwell, L. (1992) *Cell* **71,** 543–546
5. Elledge, S. J. (1996) *Science* **274,** 1664–1672
6. Weinert, T. (1998) *Curr. Opin. Genet. Dev.* **8,** 185–193
7. Zhou, B. B., and Elledge, S. J. (2000) *Nature* **408,** 433–439
8. Lieberman, H. B., Hopkins, K. M., Nass, M., Demetrick, D., and Davey, S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13890–13895
9. Enoch, T., and Nurse, P. (1990) *Cell* **60,** 665–673
10. Enoch, T., Carr, A. M., and Nurse, P. (1992) *Genes Dev.* **6,** 2035–2046
11. al-Khodairy, F., and Carr, A. M. (1992) *EMBO J.* **11,** 1343–1350
12. al-Khodairy, F., Fotou, E., Sheldrick, K. S., Griffiths, D. J., Lehmann, A. R., and Carr, A. M. (1994) *Mol. Biol. Cell* **5,** 147–160
13. Rowley, R., Subramani, S., and Young, P. G. (1992) *EMBO J.* **11,** 1335–1342
14. Cimprich, K. A., Shin, R. B., Keith, C. T., and Schreiber, S. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 2850–2855
15. Kostrub, C. F., Knudsen, K., Subramani, S., Enoch, T., Kostrub, C. F., Knudsen, K., Subramani, S., and Enoch, T. (1998) *EMBO J.* **17,** 2055–2066
16. Parker, A. E., Van de Weyer, I., Laus, M. C., Verhasselt, P., and Luyten, W. H. (1998) *J. Biol. Chem.* **273,** 18340–18346
17. Udell, C. M., Lee, S. K., and Davey, S. (1998) *Nucleic Acids Res.* **26,** 3971–3978
18. Volkmer, E., and Karnitz, L. M. (1999) *J. Biol. Chem.* **274,** 567–570
19. St. Onge, R. P., Udell, C. M., Casselman, R., and Davey, S. (1999) *Mol. Biol. Cell* **10,** 1985–1995
20. Hang, H., and Lieberman, H. B. (2000) *Genomics* **65,** 24–33
21. Burtelow, M. A., Roos-Mattjus, P. M., Rauen, M., Bahendure, J. R., and Karnitz, L. M. (2001) *J. Biol. Chem.* **276,** 25903–25909
22. Caspari, T., Dahlen, M., Kanter-Smoler, G., Lindsay, H. D., Hofmann, K., Papadimitriou, K., Sunnerhagen, P., and Carr, A. M. (2000) *Mol. Cell. Biol.* **20,** 1254–1262
23. Thelen, M. P., Venclovas, C., and Fidelis, K. (1999) *Cell* **96,** 769–770
24. Venclovas, C., and Thelen, M. P. (2000) *Nucleic Acids Res.* **28,** 2481–2493
25. Bravo, R., Frank, R., Blundell, P. A., and Macdonald-Bravo, H. (1987) *Nature* **326,** 515–517
26. Krishna, T. S., Kong, X. P., Gary, S., Burgers, P. M., and Kuriyan, J. (1994) *Cell* **79,** 1233–1243
27. Rauen, M., Burtelow, M. A., Dufault, V. M., and Karnitz, L. M. (2000) *J. Biol. Chem.* **275,** 29767–29771
28. Mossi, R., and Hubscher, U. (1998) *Eur J Biochem.* **254,** 209–216
29. Burtelow, M. A., Kaufmann, S. H., and Karnitz, L. M. (2000) *J. Biol. Chem.* **275,** 26343–26348
30. Edwards, R. J., Bentley, N. J., and Carr, A. M. (1999) *Nat. Cell Biol.* **1,** 393–398
31. Matsuoka, S., Rotman, G., Ogawa, A., Shiloh, Y., Tamai, K., and Elledge, S. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 10389–10394
32. Cortez, D., Wang, Y., Qin, J., and Elledge, S. J. (1999) *Science* **286,** 1162–1166
33. Kim, S. T., Lim, D. S., Canman, C. E., and Kastan, M. B. (1999) *J. Biol. Chem.* **274,** 37538–37543
34. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998) *Science* **281,** 1674–1677
35. Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998) *Science* **281,** 1677–1679
36. Tibbetts, R. S., Cortez, D., Brumbaugh, K. M., Scully, R., Livingston, D., Elledge, S. J., and Abraham, R. T. (2000) *Genes Dev.* **14,** 2989–3002
37. Chen, J. (2000) *Cancer Res.* **60,** 5037–5039
38. Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. (1999) *Genes Dev.* **13,** 152–157
39. Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D. A., Smith, S., Uziel, T., Sfez, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patanjali, S. R., Simmons, A., Clines, G. A., Sartiel, A., Gatta, R. A., Chessa, L., Sandal, O., Lavin, M. F., Jaspers, N. G. J., Taylor, A. M. R., Arlett, C. F., Miki, T., Weissman, S. M., Lovett, M., Collins, F. S., and Shiloh, Y. (1995) *Science* **268,** 1749–1753
40. Chen, M. J., Lin, Y. T., Lieberman, H. B., Chen, G., and Lee, E. Y. (2001) *J. Biol. Chem.* **276,** 16580–16586
41. Komatsu, K., Miyashita, T., Hang, H., Hopkins, K. M., Zheng, W., Cuddeback, S., Yamada, M., Lieberman, H. B., and Wang, H. G. (2000) *Nat. Cell Biol.* **2,** 1–6
42. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) *Mol. Cell. Biol.* **8,** 466–472
43. Fang, G., Yu, H., and Kirschner, M. W. (1998) *Mol. Cell* **2,** 163–171
44. Endicott, J. A., Noble, M. E., and Tucker, J. A. (1999) *Curr. Opin. Struct. Biol.* **9,** 738–744
45. Holmes, J. K., and Solomon, M. J. (1996) *J. Biol. Chem.* **271,** 25240–25246
46. Murray, J. M., Carr, A. M., Lehmann, A. R., and Watts, F. Z. (1991) *Nucleic Acids Res.* **19,** 3525–3531

DNA Damage-Dependent and -Independent Phosphorylation of the hRad9 Checkpoint Protein

by

Blair D. A. Besley

A thesis submitted to the Department of Biochemistry

in conformity with the requirements for

the degree of Master of Science

Queen's University

Kingston, Ontario, Canada

March 2002

For Arnold and Diane Besley,
and their unquestioning support of all that I do.

Abstract

The G2 DNA damage checkpoint prevents the initiation of mitosis in response to DNA damage or the inhibition of DNA replication, ultimately preventing the propagation of mutations. It has been demonstrated that hRad9 is the ortholog of fission yeast Rad9 which is essential for G2 checkpoint activity. It was observed that hRad9 exists in three distinct complexes, which are invariant in their relative abundance with respect to cell cycle position. However, it was also observed that hRad9 is phosphorylated specifically at G2/M of the cell cycle in HeLa cervical carcinoma cells, the first indication of any G2 specific alteration of hRad9. The cell cycle-dependent phosphorylation of hRad9 is distinct from the DNA damage inducible phosphorylation attributed to the ATM kinase, and occurs independently of the ATM dependent phosphorylation. Furthermore, in karyotypically-normal hTERT-RPE1 retinal epithelial cells it was observed that the cell-cycle specific phosphorylation of hRad9 could only be induced by treatment with DNA damage stimuli.

Targeted mutagenesis of 13 serine, threonine, and tyrosine residues conserved between the fission yeast, murine, and human Rad9 orthologs failed to identify the specific sites of hRad9 cell cycle phosphorylation. Furthermore, none of the hRad9 point mutants interfered with normal cell cycle progression, or interfered with ionizing radiation-induced G2 arrest. The cell cycle-dependent phosphorylation of hRad9 was also not observed to effect the formation of extraction resistant forms of hRad9 in response to DNA damage or replication inhibition.

Acknowledgements

I would like to thank my supervisor Dr. Scott Davey for taking me into his laboratory and for his support and guidance throughout my masters project. I would also like to thank my supervisory committee, Dr. Alan Mak, Dr. Graham Côté, Dr. Martin Petkovich, as well as all members of the Davey Lab, past and present, for their helpful discussions and suggestions. In addition, I would like to thank Mr. Derek Schultze for his assistance with the flow cytometry described in this thesis. This work was supported by grants from the Canadian Institutes of Health Research and a U.S. Army Breast Cancer Research Pre-doctoral Studentship (Award #, DAMD17-98-1-8080).

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AEBSF	4-(2-Amino-ethyl)-benzenesulfonyl fluoride
ATM	Ataxia Telangiectasia Mutated Kinase
ATP	adenosine tri-phosphate
ATR	ATM and Rad3 Related Kinase
bp	base pair
BSA	bovine serum albumin
CDK	cyclin dependent kinase
cDNA	complementary DNA
CIP	calf intestinal phosphatase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic Acid
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
β-GP	β-glycerophosphate
Gy	Gray; unit of radiation equivalent to 100 rads
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HRP	horseradish peroxidase
HU	hydroxyurea
IgY	immunoglobulin Y
IR	ionizing radiation
kb	kilobase pair
kDa	kilo Dalton
LB	Luria-Bertani
MDa	mega Dalton
M	moles per liter
mM	millimoles per liter
NaF	sodium fluoride
nm	nano meter
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PI	propidium iodide
PNK	polynucleotide kinase
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet radiation

Chapter 1

Introduction

1.1 Cell Cycle Checkpoints

The cell has a number of mechanisms that ensure the proper chronology of cellular events and the fidelity of its genome; these mechanisms have been termed cell cycle checkpoints (1-3). In any single given oscillation of the cell cycle, the completion of certain cellular events prior to the initiation of other later events is essential for orderly cell division. Furthermore, a cell must ensure that those events involving the replication and transmission of its genome to its offspring are executed with optimal fidelity. The cell cycle checkpoint response is a diverse response involving cell cycle arrest (3, 4), the induction and repression of DNA damage inducible genes (5-10), and the up regulation of DNA repair and apoptotic pathways (4, 10-14).

Cell cycle checkpoints regulate entry into S-phase and the entry and exit of mitosis in response to a number of cytotoxic stimuli (6, 15-17). Checkpoints serve to regulate cell cycle transitions by arresting the cell cycle at the G1/S (6, 15, 18)and G2/M transitions (19-23) in response to DNA damage, to allow for DNA repair prior to DNA replication and mitosis (1, 3, 19, 20, 24, 25). Furthermore, cell cycle checkpoints serve to arrest DNA replication within S-phase in response to DNA damage (19, 26-31). In addition, the S-phase checkpoint has been demonstrated to prevent the onset of mitosis until DNA replication can be properly completed in fission yeast (21-23) as well as in the mammalian system (19, 31, 32). Cell cycle checkpoints also function to regulate entry into mitosis in

response to cell volume, and prevent mitotic exit in response to improperly segregated sister chromatids (17, 33, 34). Furthermore, in addition to their role in monitoring the mitotic cell cycle, checkpoint proteins have a demonstrated role in the maintenance of telomeres (35-42), and the regulation of the meiotic cell cycle in fission yeast (43, 44), budding yeast (45, 46) and mammalian cells (44, 47).

Cell cycle checkpoint pathways serve to prevent progression of the cell cycle at G1/S, within S-phase, G2/M and within mitosis in response to any event which may result in non-viable progeny, or the propagation of mutations within a given population of cells.

1.2 DNA Damage Checkpoints

It has been shown that the length of cell cycle arrest at the G1/S and G2/M transitions induced by DNA damage occurs in a dose dependent fashion (3, 6, 13, 19, 48). The dose dependency of checkpoint activity presumably occurs to facilitate increasing amounts of DNA repair being required due to an elevated incidence of DNA damage (1, 3, 24). Examples of DNA damage stimuli which induce arrest of the cell cycle include: double and single stand breaks induced by X- or γ- radiation (49, 50) and chemical modifications of DNA caused by compounds that methylate or oxidize DNA (51, 52). Furthermore, UV-induced base mutations, which block DNA replication (53, 54), as well as chemicals which inhibit DNA replication like the ribonucleotide reductase inhibitor hydroxyurea have been demonstrated to activate cell cycle checkpoint pathways

in eukaryotes (21-23, 55). Cell cycle checkpoint pathways have an important role in the normal life cycle of a healthy cell; checkpoints are proposed to survey for self-inflicted genomic wounds like stalled replication forks (56), recombination intermediates (46, 57), and naturally occurring base mismatches (58, 59). The importance of checkpoint proteins in the development and normal life cycle of the somatic cell is illustrated by the embryonic-lethal phenotype and massive genomic instability observed in transgenic mice deleted for the *hus1* (32), *ATR* (60), and *chk1* (61) genes.

1.2.1 The G1/S Checkpoint

While this thesis deals with the S and G2/M checkpoints, the classic example of eukaryotic checkpoint control is the role of p53 and the G1 DNA damage checkpoint (11). Therefore, to place the importance of an understanding of the S-phase and G2/M checkpoints in the proper context, a description of the G1 checkpoint is provided to illustrate the importance of an understanding of the G2/M checkpoint in treatment of cancers which harbor mutant p53 alleles.

As mentioned previously, initiation of DNA replication is governed by the G1/S checkpoint (6, 13, 15, 16). Ultimately, regulation of the G1/S transition is dependent upon the sequential activation of the CDK6/cyclin D, CDK4/cyclin D, CDK2/cyclin E and CDK2/cyclin A serine/threonine kinase complexes (62-66). Activated cyclin/CDK complexes phosphorylate and thereby inhibit the retinoblastoma (Rb) tumor suppressor protein (7, 63, 64, 67). The normal function of Rb is to bind the E2F family of transcription factors, inhibiting their

transcriptional acitivity (68-71). However, Rb phosphorylation by cyclin/CDK complexes results in Rb no longer being able to bind and inhibit the E2F transcription factors, allowing transcription of E2F responsive genes and induction of DNA replication (7, 72-74). The p53 tumor suppressor protein is essential for the G1/S checkpoint, as activated p53 prevents activation of CDK/cyclin complexes in response to DNA damage stimuli as part of its array of action (8, 75-77).

The best characterized example of a defect in proper checkpoint function resulting in a loss of cell cycle control and carcinogenesis is the p53 tumor suppressor protein. Sporadic mutations in p53 occur in a majority of human cancers (11, 78, 79), and germline mutations of the *p53* gene have been demonstrated to cause Li-Fraumeni cancer susceptibility syndrome (80-82). The p53 protein is a transcription factor whose activity is increased by elevation of p53 levels in response to DNA damage stimuli (6, 11, 83).

Cells which express loss of function or dominant negative forms of p53 have a compromised G1/S checkpoint (13, 15) and do not undergo apoptosis (84-86) in response to exposure to DNA damage stimuli, and as a result, DNA replication occurs in cells which are treated with DNA damage stimuli resulting in the potential fixation of mutations.

Nevertheless, it should be noted that *p53* null cells which lack a functional G1/S checkpoint will still arrest at the G2/M checkpoint upon treatment with genotoxic agents (6), and do not display enhanced sensitivity to IR or chemotherapeutic agents (16). Thus, the understanding of the G2/M and S-

phase checkpoints is of particular importance in the etiology of human cancer. Furthermore, the study of the regulation of the G2/M and S-phase checkpoints is important, as the inhibition of these checkpoints would be of high therapeutic utility in the sensitization of p53-mutant cancer cells to radiation treatment and chemotherapy (30, 87-91).

1.3 Regulation of the G2/M Transition.

The G2 phase of the cell cycle exists to ensure that the cell has completed all the tasks required for it to sub-divide into two daughter cells during mitosis. The cell must ensure complete DNA replication (19, 21-23), that the cell has increased in volume sufficient for two cells (33), and that DNA damage does not exist which will result in non-viable progeny (1-3, 19, 21-23).

Progression from G2 into mitosis, similar to the G1/S transition, is ultimately regulated by the activation of a cyclin dependent kinase complex. The Cdc2/cyclin B complex responsible for the regulation of the G2/M transition in humans and in fission yeast was originally described as the M-phase promoting factor (MPF) in frog oocytes as a factor capable of inducing entry into mitosis (92-99). The activation of Cdc2/cyclin B complexes is essential for progression through mitosis as Cdc2/cyclin B has been demonstrated to phosphorylate important structural components of the cell. Cdc2/cyclin B substrates include nuclear lamina, caldesmon, and the kinesin-like Eg5 protein and their phosphorylation drives entry into mitosis by the breakdown of the cellular structures (100-103).

1.3.1 Cdc2/Cyclin B, Cdc25, and Wee1

The regulation of Cdc2 occurs on two levels: Regulatory cyclin B binding and inhibitory phosphorylation. Cyclin B is a regulatory subunit of the Cdc2 kinase, and is essential for its activity (104-106). Cyclin B levels are regulated by cell cycle position and DNA damage. Regulation of cyclin B occurs at the mRNA and protein levels, with cyclin B levels peaking at G2/M of the cell cycle, giving the cell cycle specific activity of Cdc2 (105-109). In addition, cyclin B levels also decrease in response to ionizing radiation, concurrent with checkpoint activity (110).

However, cyclin B binding is not sufficient for activation of Cdc2. The human Cdc2 kinase itself is inhibited by phosphorylation on three negative regulatory sites, threonine 14, tyrosine 15, and threonine 161 (93, 111-113). Inhibition of Cdc2 activity by phosphorylation blocks progression through G2/M of the cell cycle (113, 114). The major mode of Cdc2 inhibition is by phosphorylation at tyrosine 15 by the Wee1 tyrosine kinase, a target of the G2/M checkpoint response (115, 116). Mutation of Cdc2 inhibitory phosphorylation sites results in constitutive Cdc2 activation, resulting in cells being unable to complete DNA replication prior to entry into mitosis or arrest at the G2/M transition in the presence of DNA damage (117-119).

The Cdc25C phosphatase works in opposition to the Wee1 kinase in the regulation of the Cdc2/cyclin B complex. The Cdc25C phosphatase is a dual specificity phosphatase that is a member of a family of phosphatases (Cdc25A,

B, and C) in mammalian cells that are responsible for the regulation of cyclin dependent kinase complexes (112, 120-122). Cdc25C dephosphorylates threonine 14 and tyrosine 15 in human Cdc2 (112, 120-122). The dephosphorylation of Cdc2 results in its activation and the initiation of mitosis (112, 120-122). Moreover, Cdc2 exists in an autocatalytic loop with Cdc25C. Once activated, Cdc2 phosphorylates Cdc25C resulting in a higher Cdc25C activity and more Cdc2 molecules being activated (123). Furthermore, mutation of Cdc2 phosphorylation sites in Cdc25C results in cells being arrested at G2/M (123).

1.4 The DNA Replication Checkpoint and the G2 DNA damage checkpoint

The intra-S phase checkpoint is responsible for the arrest of DNA replication in response to DNA damage, and prevention of the onset of mitosis until completion of DNA replication (21-23, 29, 31). The G2 checkpoint was originally proposed to be responsible for arresting cells at the G2/M transition in response to DNA damage inflicted in G2 phase of the cell cycle (1, 21-23). Both checkpoints in humans and fission yeast are demonstrated to act through the ultimate inhibition of Cdc2/cyclin b (21-23, 93, 124), however it is becoming apparent that how these mechanisms achieve their eventual inhibition of Cdc2/cyclin B is more sophisticated than first hypothesized.

In their work, Xu *et al.* describe two distinct G2/M checkpoints at work in mammalian cells, in addition to the intra S-phase checkpoint (19); it was shown that cells which were in G2 and S phase of the cell cycle arrested immediately in

response to IR (19). However, cells that were deleted for the *ATM* gene did not arrest immediately in response to IR while in S-phase or G2 (19). Instead, *ATM*$^{-/-}$ cells irradiated in S-phase subsequently arrested in G2 for an extended period of time, and cells irradiated in G2 did not arrest at all in response to ionizing radiation (19). Xu *et al.* term the immediate arrest in G2 to be the "early" G2 checkpoint and the arrest in G2 after irradiation in G1 or S-phase to be"G2-accumulation" (19). This paradoxical result suggests that the immediate arrest in S and G2 phase (or "early" G2 checkpoint) is the same mechanism, with the "G2 accumulation" being another checkpoint mechanism. Interestingly, the "G2 accumulation" phenomena described by Xu *et al.* (19) is more reminiscent of the S-phase and G2 checkpoints observed in genetic analyses in fission yeast (21-23). The overlap of the intra-S, "early-G2" and "G2-accumulation" pathways, and which factors participate in these distinct pathways will be an area of considerable interest in the near future.

1.4.1 Checkpoint Proteins

Studies in the genetically tractable fission and budding yeasts have revealed a series of genes, that when deleted, prevent cells from arresting in response to DNA damage or replication inhibition, resulting in a hyper sensitivity to DNA damaging agents and replication inhibitors (1-3, 21-24).

The initial description of a G2 checkpoint gene was made in budding yeast by Weinert and Hartwell in their description of *S. cerevisae RAD9* (1, 3, 24). In their work, it was shown that disruption of the *RAD9* gene in *S.cerevisae* prevented

cell cycle arrest at the G2/M transition in response to ionizing radiation, resulting in cell death in subsequent cell divisions (1, 24). Furthermore, Hartwell and Weinert also demonstrated increased cell survival could occur in irradiated populations of *RAD9* deficient cells that were treated with a microtubule poison that arrested cells at G2 for several hours (24). Hartwell and Weinert's description of *S.cerevisae RAD9* was the basis for the concept of cell cycle checkpoints (1-3, 24, 125). However, for the purposes of this thesis the budding yeast DNA damage checkpoint pathway has been omitted as the budding yeast cell cycle is spatially and temporally very different from that found in mammalian cells or fission yeast.

1.4.1 The Checkpoint Rads and the Proposed Functions of the Human Homologs of the Checkpoint Rad Proteins.

Genetic screens in the fission yeast *Schizosaccharomyces pombe* revealed a set of genes (*rad1*$^+$, *rad3*$^+$, *rad9*$^+$, *rad17*$^+$, *rad26*$^+$, *and hus1*$^+$) that when deleted made cells hyper-sensitive to DNA replication inhibitors and to DNA damage (21-23). These six genes have been termed the '*checkpoint rad*' genes, and are functionally conserved in all eukaryotes, including humans (Table 1) (21-23, 126-129). Herein, I will refer to the *checkpoint rad* genes and their protein products using the nomenclature of their human forms.

The powerful genetic analyses allowed by the fission yeast system began to reveal functional information about the checkpoint proteins. It was determined that the checkpoint Rads had dual roles in the activation of the S-phase and G2 DNA damage checkpoints (21-23). Furthermore, the presence of the *checkpoint*

Table 1: The *checkpoint rad* genes and the effector kinases in fission yeast and their homologs in budding yeast, and humans.

	S.pombe	*S.cerevisae*	*H.sapiens*
PCNA-like proteins	*rad9*	*DDC1*	*hRad9*
	rad1	*RAD17*	*hRad1*
	hus1	*MEC3*	*hHus1*
RFC-like proteins	*rad17*	*RAD24*	*hRad17*
Coiled-coil proteins	*rad26*	*DDC2*	*ATRIP*
PI-3 Kinase like proteins	*rad3*	*MEC1/TEL1*	*ATR/ATM*
Effector Kinases	*cds1*	*RAD53*	*Chk2*
	chk1	*CHK1*	*Chk1*

rads was determined to be required for the activation of the downstream targets, including Cdc25, and Cdc2; suggesting that the checkpoint rads were involved in Cdc2 dependent G2 arrest (21-23, 130-132).

1.4.2 rad3, rad26, ATM, ATR and ATRIP

The fission yeast protein Rad3 is a serine/threonine protein kinase containing a PI-3-kinase related catalytic domain. The Rad3 protein complexes with, and phosphorylates, Rad26, a coiled-coil domain containing protein (133). Phosphorylation of Rad26 by Rad3 is independent of the remaining four checkpoint proteins (Rad1, Rad9, Rad17, and Hus1), however the other checkpoint proteins are required for downstream transduction of the checkpoint signal through Rad3 to Cdc2 (134, 135). It has been suggested that Rad3-Rad26 exists in a checkpoint pathway distinct from the other checkpoint Rads as Rad3 is required to initiate, but not maintain the checkpoint response (133, 134).

Consistent with results in fission yeast two PI-3 related serine/threonine kinases have subsequently been identified to play similar roles in human cells as Rad3 does in fission yeast. The two kinases, ATM and ATR, are of fundamental importance in the human DNA damage response and checkpoint signaling. Substrates of ATM and ATR include a large number of proteins involved in the checkpoint response and DNA repair including, p53 (28, 136-138), MDM2 (139, 140), BRCA1 (141, 142), hRad17 (143-145), hRad9 (146), histone H2AX (147, 148), Chk1 (149-152), Chk2 (31, 153-155), and Nbs1 (29). Furthermore, there is

evidence indicating ATM and ATR themselves bind DNA, thus being directly being responsible for genome surveillance (143, 156, 157).

ATM and ATR substrate specificities have been determined in studies using oriented peptide libraries, and the mapping of physiological phosphorylation sites. It has been determined that ATM and ATR phosphorylate serine and threonine residues that are followed by a glutamine residue ([ST]Q motif) (29, 158, 159) as they phosphorylate an overlapping range of substrates, albeit usually one kinase is usually favored over the other, suggesting a more sophisticated mechanism of substrate recognition than just the simple [ST]Q motif.

The ATM kinase is thought to have a very wide range of roles in the cell. Germline homozygous mutations in the *ATM* gene have been demonstrated to be the causative factor in the cancer susceptibility syndrome ataxia telangiectasia (AT) (48, 160, 161). AT patients are shown to have immune deficits, a loss of motor control, exhibit high frequencies of cancer, and to be infertile, phenotypes that have been recapitulated in $ATM^{-/-}$ transgenic mice (48, 162). In addition, cells derived from AT patients and from $ATM^{-/-}$ mice are demonstrated to be deficient in multiple cell cycle checkpoints, and are extremely sensitive to IR (18, 19, 48, 163, 164). It has been shown that heterozygous carriers of a mutant *ATM* allele have been demonstrated to exhibit heightened cancer susceptibility (165-168); however, this hypothesis is a matter of some controversy (169-171). It should be noted, that ATM substrates are still

phosphorylated in *ATM* deficient cells, however with delayed kinetics by what is thought to be ATR (28, 136, 137).

The G2 checkpoint role of ATR is demonstrated in cells that are transfected with a kinase-inactive form of ATR (ATR-ki). Reports have demonstrated that addition of ATR-ki sensitizes cells to all forms of DNA damage stimuli, including IR, UV, and replication blockages, and diminishes the G2 checkpoint response induced by radiation (172). In addition, $ATR^{-/-}$ mice exhibit peri-implantation embryonic lethality, with $ATR^{-/-}$ blastocysts dying under culture conditions with an abrogated G2/M checkpoint, resulting in the premature entry of cells into mitosis, aberrant chromosome condensation and nuclear fragmentation indicative of a mitotic catastrophe (173). Consistent with work in transgenic mice, a conditional $ATR^{flox/-}$ cell line has been described (129). It has been demonstrated that after excision of the second ATR allele by Cre-recombinase, $ATR^{-/-}$ cells persist in culture for a maximum of five days before dying (129). The finding of $ATR^{-/-}$ lethality in somatic cells indicates that the ATR kinase is involved in cell viability in undamaged cells, possibly in surveillance of the genome during replication, and that the null mouse phenotype is probably not developmental in nature.

The human Rad26 homolog, called ATRIP, was recently identified by mass spectrometry in ATR immunoprecipitates, (129). ATRIP is also a substrate of ATR, similar to the relationship between Rad26 and Rad3 (129, 134). Interestingly, ATRIP and ATR levels in the cell are cooperatively regulated; the addition of small interfering RNA (siRNA) directed against ATRIP reduced levels of both proteins. Furthermore, ATRIP levels were reduced in the $ATR^{flox/-}$

conditional cell line upon excision of the second *ATR* allele (129). ATR substrate specificity is regulated by the other checkpoint proteins (143, 174), consistent with work in fission yeast. In *Hus1*$^{-/-}$ mouse embryonic fibroblasts, genotoxin induced Chk1 and hRad17 phosphorylation is abolished, indicative of a blockade of ATR activity (174).

1.4.3 The hRad17 RFC complex

The hRad17 checkpoint protein is thought to be a key initiator of the G2 DNA damage checkpoint (144). The hRad17 orthologs in fission yeast and human cells have limited sequence homology with the DNA polymerase accessory factor replication factor C (RFC) (175). RFC is a heteropentameric complex consisting of one large subunit (p140, 140 kDa) and four smaller subunits (p36, p37, p38 and p40, 36, 37, 38 and 40 kDa respectively) (176). RFC serves to load the homotrimeric proliferating cell nuclear anigen (PCNA) onto DNA factor during DNA replication (177-179).

Interestingly, hRad17 has been demonstrated to exist in complex with the four small RFC subunits (180, 181), with hRad17 replacing p140 in a large proportion of cellular RFC complexes (181). *In vitro*, hRad17-RFC complexes have been shown to preferentially bind to single stranded DNA (181); stimulating hRad17 ATPase activity, consistent with a RFC-like Rad17 activity (181). In addition, there is *in vivo* data in both fission yeast and mammalian cells suggesting that hRad17 binds chromatin (143, 180, 181). Furthermore, it has been demonstrated that chromatin bound hRad17 is phosphorylated by ATR and ATM

at two sites (Serine 635 and 645) in response to DNA damage (144, 145, 182). Another report describing hRad17 phosphorylation indicates that it is greatly preferred by ATR compared to ATM, and that the phosphorylation is regulated in a cell cycle dependent manner occurring in the S and G2-phases of the cell cycle (145). It has also been demonstrated that when a mutant hRad17 lacking its ATM/ATR phosphorylation sites is expressed cells can no longer arrest at the G2/M transition in response to ionizing radiation treatment (144). The hRad17 protein has been shown to interact with three other checkpoint proteins: hRad9, hRad1 and hHus1 (183).

1.4.4 The hRad9 checkpoint protein and the 9-1-1 complex

The hRad9, hRad1 and hHus1 checkpoint proteins are the enigma of the checkpoint response, and despite controversial, and sometimes conflicting, reports ascribing 3'-5' exonuclease activity to both hRad9 (184) and hRad1 (44, 185), the hRad9, hRad1, and hHus1 proteins are without a truly defined function or downstream target.

The weight of evidence has shown that the hRad9, hRad1 and hHus1 exist in a ternary complex (9-1-1 complex) with 1:1:1 stoichiometry (186-189). In addition, The hRad9 protein has been demonstrated to have a number of additional interacting partners including histone deacetylase 1 (190), topoisomerase binding protein II (191), the anti-apoptotic Bcl-2 and Bcl-X proteins (192).

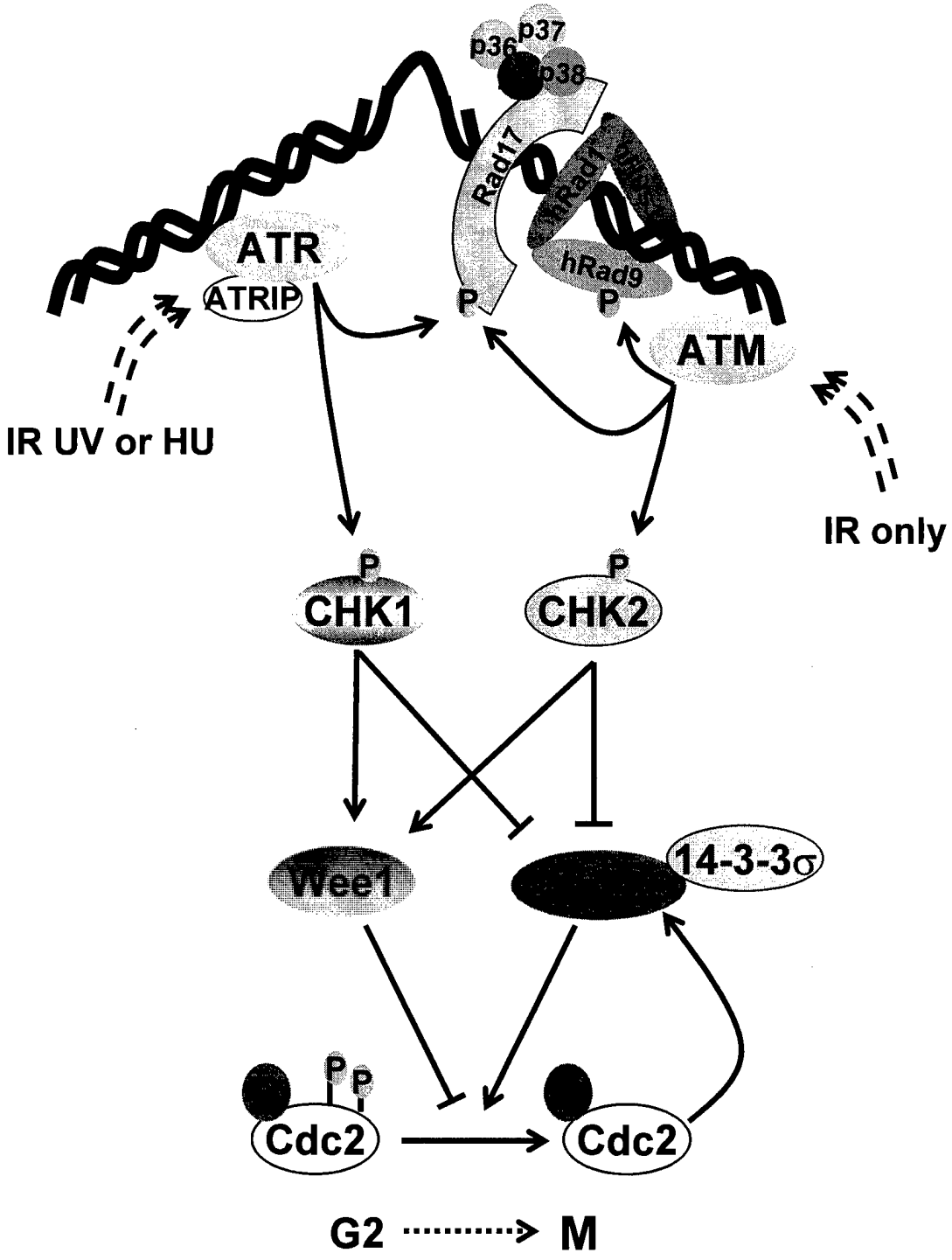
Each of hRad1, hHus1, and hRad9 has been shown to exhibit limited sequence homology to PCNA, (175). However, hRad9 (391 residues) is much larger than PCNA (261 residues), with the PCNA homology in hRad9 being found in the N-terminal two-thirds of hRad9 (175). The C-terminal 111 residues of hRad9 contain an extensively phosphorylated domain of unknown function (193). It has been demonstrated that the C-terminus of hRad9 is phosphorylated at five cyclin dependent kinase consensus sites, and is phosphorylated in-vitro by CDK4, CDK2, and CDC2 (193) (St.Onge and Davey, Unpublished Results). The extensive phosphorylation of hRad9 results in a complex banding pattern by SDS-PAGE, with most of the hRad9 in the cell visible at approximately 62 kDa (hRad9α). Partially phosphorylated forms of hRad9 are visible by SDS-PAGE between 47 kDa and 62 kDa (hRad9β) when large amounts of hRad9 are loaded onto a gel, when hRad9 is over-expressed, or when hRad9 is immunoprecipitated from cells (193). It has been demonstrated that hRad9 is phosphorylated by the ATM kinase in response to ionizing radiation (146, 193), resulting in a subtle electrophoretic mobility shift in the hRad9 protein by SDS-PAGE (hRad9γ), running slightly slower than the hRad9α form.

Each of the 9-1-1complex proteins are localized to the nucleus of the cell, and have been proposed to play a role in surveillance of the genome (186, 194, 195). The PCNA homology of the 9-1-1 complex and the similarities of the hRad17 complex to the RFC complex leads to the tempting speculation that hRad17 loads the 9-1-1 complex proteins onto DNA as a heterotrimeric sliding clamp at

sites of DNA damage, initiating checkpoint activity by acting as the most proximal end of the DNA damage checkpoint mechanism (175).

Empirical and theoretical support of the hypothesis that hRad17 is responsible for loading the 9-1-1 complex onto DNA is considerable. Data from molecular modeling studies has demonstrated that hHus1, hRad1, and the C-terminal 280 amino acids of hRad9 can all be threaded onto the known PCNA structure and model favorably in a heterotrimeric clamp structure (175). Furthermore, the 9-1-1 complex has been shown to bind to chromatin in response to DNA damage or replication inhibition (143, 195). Interactions between hRad17 and the 9-1-1 complex proteins have been demonstrated by co-immunoprecipitation (183), with a direct pairwise interaction being demonstrated by yeast-two-hybrid between hRad17 and hRad1 (196). In addition, the interaction between the hRad17 and hRad1 proteins is enhanced in response to IR *in vivo* (144), suggesting that hRad17 is responsible for loading hRad9-containing complexes onto chromatin in response to DNA damage through its interaction with hRad1, consistent with the RFC and PCNA homologies of the respective hRad17 and 9-1-1 complexes. In support of this hypothesis, it has been shown that hRad9 chromatin binding in response to genotoxins is reduced in cells that have had their hRad17 levels greatly reduced by siRNA interference directed against hRad17 (143). The overall model and relationships between the 9-1-1 complex and hRad17-RFC awaits rigorous structural analysis. The proposed relationship between the 9-1-1 and hRad17 complexes, and the rest of the checkpoint machinery is detailed in Figure1.

Figure 1. Proposed model of the DNA replication and G2 DNA damage checkpoint in mammalian cells. DNA damage in the form of IR, UV, or replication arrest results in Rad17 dependent loading of 9-1-1 complexes onto damaged DNA. Phosphorylation of hRad9 and hRad17 is catalyzed by ATM and ATR, and is dependent upon the type of damage inflicted upon cells. ATM/ATR dependent phosphorylation of Rad17-RFC_{1-4} and the effector kinases Chk1 and Chk2 is dependent on 9-1-1 loading onto chromatin. Activation of Chk1 and Chk2 results in the phosphorylation of the Cdc25 phosphatases and Wee1 dual specificity kinase, and a concerted inhibition of Cdc2. Phosphorylation of Cdc25 results in its binding 14-3-3 proteins and relocalization to the cytoplasm where it is unable to remove inhibitory phosphates from Cdc2. Furthermore, activated Wee1 phosphorylates of Tyrosine-15 of Cdc2-cyclin B complexes, inhibiting Cdc2.


p36
p37
p38
Rad17
hRad1
hRad9
P
P
ATR
ATRIP
ATM
IR UV or HU
IR only
P
CHK1
P
CHK2
Wee1
14-3-3σ
P
P
Cdc2
Cdc2
G2
M

Further genetic support for the 9-1-1 complex acting at the proximal end of the checkpoint signaling network is given from work in fission yeast and from studies in transgenic mice. In fission yeast, Rad3 is incapable of phosphorylating its downstream targets in the absence of any of the other five checkpoint Rad proteins (134). In addition, it has been revealed in *hus1*$^{-/-}$ mouse embryonic fibroblasts that Hus1 is required for phosphorylation of Rad17 and Chk1 by the ATR kinase (174). Furthermore, transgenic *hus1*$^{-/-}$ mice are embryonic lethal with primary cells derived from *hus1*$^{-/-}$ mice exhibiting chromosomal abnormalities (32). The checkpoint effect of the *hus1*$^{-/-}$ genotype could not be accurately assessed, as primary cells derived from *hus1*$^{-/-}$ mice were unable to proliferate in culture unless crossed into a *p21*$^{-/-}$ background (32).

1.4.5 Effector Kinases Chk1 and Chk2/Cds1

Chk1 and Chk2 link the checkpoint machinery to cell cycle regulation and DNA repair. The serine/threonine effector kinases Chk1 and Chk2/Cds1 (human/fission yeast) have been demonstrated to relay checkpoint signals to a diverse array of downstream targets from the genome surveillance machinery, including the transmission of signals to the cell cycle machinery, and ultimately Cdc2 (22, 26, 131, 132, 135, 154, 164, 197, 198). The concerted action of Cdc25C and Wee1 on Cdc2/cyclin B complexes is subject to checkpoint-mediated regulation via Chk1 and Chk2.

Chk1 and Chk2/Cds1 are structurally unrelated and appear to participate in distinct checkpoint pathways based upon genetic analyses in fission yeast and

transgenic mice (22, 26, 131, 132, 135, 154, 164, 197, 198). However, Chk1 and Chk2/Cds1 have been shown to have overlapping substrate specificities, suggesting that they function in different checkpoint pathways to elicit checkpoint arrest and DNA repair by the same end-pathway. Substrates of Chk1 and Chk2/Cds1 include the checkpoint regulating Cdc25 phosphatases and Wee1 kinase (27, 135, 154, 198, 199). Upon activation of the G2 or S-phase checkpoints, Cdc25C and Wee1 are directly phosphorylated by one of the checkpoint effector kinases: Chk1 or Chk2 (197, 200-204). Phosphorylation of Serine 216 of Cdc25C results in binding of the 14-3-3 protein 14-3-3σ, relocalization of Cdc25C to the cytoplasm, and inhibition of its activity (199, 205-212). Concurrent with Cdc25C inhibition by 14-3-3 proteins it has also been demonstrated that phosphorylation of Wee1 results in 14-3-3 binding and an up-regulation of Wee1 kinase (213-215).

Furthermore, Chk2/Cds1 phosphorylation has been shown to stabilize p53 (216-218); interestingly, a subset of Li-Fraumeni patients have been shown to express mutant *Chk2* alleles, rather than mutant *p53* alleles, consistent with the role of Chk2 in p53 stabilization (216, 219).

Both Chk1 and Chk2/Cds1 are phosphorylated in response to DNA damage by phosphpinositide-3 related (PI-3 related) protein kinases that are closely related to the Rad3 kinase found in fission yeast. Chk2 is phosphorylated at threonine 68 in response to IR by the ATM kinase (153, 155, 220, 221). Inducible phosphorylation of Chk2 at threonine 68 has been shown to be required for efficient activation of Chk2 kinase activity in response to DNA

damage (220-222). In contrast, Chk1 is phosphorylated at multiple sites in response to IR, UV, and replication blocks by the ATR kinase (149, 150, 152). However, the phosphorylation of Chk1 does not appear to stimulate its kinase activity and is thought to mediate binding of Chk1 substrates or interacting partners. Furthermore, the Rad3-Rad26 complex in fission yeast was demonstrated to phosphorylate the Chk1 and Cds1 effector kinases in response to DNA damage (222, 223).

Fission yeast genetics has demonstrated that Cds1 is an effector of the S-phase checkpoint (26, 27, 224). The role of Cds1 in the S-Phase checkpoint is indicated by the inability of *cds1* null cells to arrest themselves in S-phase in response to DNA damage or compounds that block DNA replication (224). This is consistent with reports in mammalian cells demonstrating that Chk2 is phosphorylated and activated in response to DNA damage and, has a role in protection against radio-resistant DNA synthesis, and in a transient response to DNA damage (19, 31). It has been demonstrated that mouse embryonic stem cells deleted for *chk2* are capable of properly initiating, but not maintaining the G2 checkpoint in response to DNA damage for extended periods (216).

Meanwhile, the weight of evidence from fission yeast and mammalian cells strongly implicates Chk1 as being involved in the G2-DNA damage checkpoint delay. Chk1 kinase activity has been demonstrated to be enhanced in the S and G2-phases of the cell cycle (151). Fission yeast deleted for Chk1 are replication checkpoint proficient, but cannot arrest at, or accumulate in, G2 in response to DNA damaging agents (131, 132, 197). Furthermore, *chk1*$^{-/-}$ mice are not viable

at the blastocyst stage, dying between 3.5 and 6.5 days after fertilization with gross morphologic abnormalities in their nuclei (61). In addition, *chk1*$^{-/-}$ blastocysts cannot prevent the onset of mitosis in response to IR or replication inhibition, similar to a report describing a null *chk1* cell line created by conditional recombination (61, 150).

This thesis describes a study of the complex state of the hRad9 checkpoint protein, a description of a novel cell cycle specific phosphorylation of hRad9, and the implications for hRad9 chromatin binding.

Chapter 2

Materials and Methods

2.1 Bacterial Manipulations

All transformations of plasmid DNA for normal propagation were done in XL1-Blue chemically competent *Escherichia coli* (F'::Tn10 *proA*$^{+}$*B*$^{+}$ *lacI* ^{q} D*(lacZ) M15/recA1 endA1 gyrA96* (Nal^{r}) *thi hsdR17* ($r_K^- m_K^+$) *supE44 relA1 lac*) (225). Site-directed mutagenesis reactions employed electro-competent, mismatch repair deficient, BMH 71-18 *mutS* null bacteria (*thi, supE, Δ(lac-proAB),* [*mut*S::Tn10][F'*proAB*, *lacI* ^{q} ZΔM15].

Both bacterial strains XL1-Blue and BMH 71-18 were propagated in LB medium (10 g/L Tyrptone/Peptone, 10 g/L NaCl, and 5 g/L Yeast Extract) supplemented with 100 μg/mL ampicillin (LBamp), or were grown on solid LB +2% Agar plates supplemented with 100 μg/mL ampicillin (LBamp plates). All liquid cultures were grown at 37°C with constant agitation; all bacteria on solid media were grown at 37°C, where indicated.

For normal transformation, chemically competent XL1-Blue bacteria (225) (100 μL) were transformed with 1-5 μL of miniprep DNA (preparation described below). Competent bacteria and DNA were mixed and incubated on ice for 10 minutes, treated at 42°C for 1 minute, and allowed to recover for 1 hour in 1 mL of LB media lacking antibiotic. After recovery, transformed bacterial cultures were diluted to 5 mL in LBamp or 20 μL was plated on LBamp plates, and grown overnight at 37°C. Liquid cultures were grown with a constant agitation of 220

rpm on a G10-Gyrotory Shaken (Edison Scientific, Edison NJ). Chemically competent XL-1-Blue bacteria (225) were generated by Kathy Kennedy.

In the initial step of site directed mutagenesis 2 μL of each mutagenesis reaction was diluted to 10 μL with sterile H_2O and transformed by electroporation into 50 μL of electro-competent BMH 71-18 bacteria. Electroporations were done using a Micro-Pulser electroporator (Bio-Rad, Hercules CA) in a 0.2 cm cuvette (Bio-Rad, Hercules CA) at 2.5 kV, with time constants between 5.0-5.7 mS. Electroporated bacteria were then allowed to recover in 500 μL of LB media without antibiotic for 1 hour at 37°C, collected by brief centrifugation at 16,000 *g* and resuspended in 5 mL of LBamp overnight. Electrocompetent BMH 71-18 bacteria were generated from a stock supplied with the Transformer Site Directed Mutagenesis Kit, consistent with the manufacturer's instructions.

2.1.1 Plasmid DNA Purification

All small scale DNA preparations of recombinant DNA (miniprep) described in this thesis propagated in either BMH 71-18 or XL-1-Blue bacteria were purified using a QIAGEN Plasmid Mini Kit (QIAGEN, Mississauga ON) according to the manufacturer's instructions.

Large-scale preparations of plasmid DNA (maxiprep) were derived from 200 mL bacterial cultures grown in LB-Amp. A single colony of XL1-Blue bacteria grown on solid media was used to inoculate 5 mL LB-amp and was grown at 37°C for 8 hours. After 8 hours, the 5 mL culture was pelleted, re-suspended in 1 mL of fresh LB-amp and used to inoculate a 200 mL culture that was grown for

16-20 hours at 37°C. Bacterial cultures were harvested at 6000g for 10 minutes in a Beckman J2-21M/E centrifuge using a JA-10 rotor (Beckman-Coulter, Mississauga ON). Plasmid DNA was purified from bacterial cultures using a QIAGEN Maxi Kit (QIAGEN) consistent with the manufacturer's instructions.

2.2 Site Directed Mutagenesis

The transformer site directed mutagenesis kit, purchased from Clontech (Palo Alto, CA) was used to introduce point mutations into the *hRad9* cDNA expressed from the pYDF-hRad9 expression construct. Oligonucleotides used in the generation of single amino acid mutations of hRad9 are described in Table 2. All oligonucleotides were purchased from Invitrogen/Gibco-Life Technologies (Burlington, ON) or Cortec DNA Service Laboratories (Kingston, ON). All oligonucleotides used for the insertion of point mutations were purchased with the 5'-phosphate added, and all oligonucleotides used for selection of mutant pyDF31-hRad9 constructs had the 5'-phosphate added by polynucleotide kinase (PNK) after purchase.

2.2.1 Oligonucleotide Phosphorylation

As indicated in the materials section, the oligonucleotides used for selection of mutant plasmids were purchased without a phosphate attached to their 5'-termini, and were phosphorylated by PNK. PNK reactions were performed in a total volume of 20 μL containing 1X PNK buffer (New England Biolabs, Beverly MA), 10 U of PNK (New England Biolabs), 1 μg of oligonucleotide, and 1 mM

Table 2: Oligonucleotides Used for Site Directed Mutagenesis and Sequencing of mutant pYDF31-hRad9 constructs.

Name	5'-3' Sequence	pYDF31-hRad9 Restriction Site Abolished
Selection #1	GCTCTAGCCCTGGAGATGAAGTGC	Xho1
Selection #2	CAAGTAGCGGCCGGTAATTCCTGATTG	Not1
S21A	CCACTCCCTGGCCCGCATCGG	N/A
Y28F	GGACGAGCTCTTCCTGGAACCC	N/A
S43A	GACGGTGAACGCCTCCCGCTC	N/A
S44A	GGTGAACTCCGCCCGCTCTGC	N/A
S46A	CTCCTCCCGCGCTGCCTATGC	N/A
T121A	GGTGCGGAAGGCTCACAACCTG	N/A
T167A	GGCTGAAGTGGCGCTGGGCATTG	N/A
T195A	AGCCATGGTGGCTGAGATGTGCC	N/A
T216A	GGTGGCCATCGCTTTCTGCCTC	N/A
T250A	CGCCATCTTCGCCATCAAGGAC	N/A
T264A	TGTCTTGGCCGCACTCTCAGAC	N/A
S341A	CGGTCCCCAGCGCGAGGAGGAAG	N/A
T2	AGAGAAGGGCAGAACAG	N/A
F4	CTTCCAGCAATACCAG	N/A

ATP. Reactions were incubated for 60 minutes at 37°C, and were subsequently quenched by heating at 65°C for 10 minutes. The final concentration of oligonucleotide in each reaction was 0.05 μg/μL. Each of the oligonucleotides designed to insert point mutations into hRad9 were diluted to 0.05 μg/mL in sterile H_2O upon receipt.

2.2.2 Mutagenesis Reactions

All buffers and enzymes, with the exception of the restriction endonucleases, were supplied by the manufacturer; the protocol described is based upon that described in the supplied instruction manual (226). All restriction enzymes used were purchased from New England Biolabs, and used in the appropriate 1X buffer consistent with the manufacturer's instructions. All digests were carried out at 37°C.

The selection oligonucleotide used to generate each hRad9 point mutant is indicated in Table 3. For each pYDF31-hRad9 point mutant generated the following were combined in an annealing reaction. Annealing reactions were made to a total volume of 20 μL containing 1X annealing buffer (20 mM Tris pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl), 0.1 μg of pYDF-hRad9 maxiprep DNA, 0.2 μg of mutagenic oligonucleotide, and 0.2 μg of selection oligonucleotide. The mixture was heated at 100°C for 5 minutes, and then mutagenic oligos were allowed to anneal by incubation of mixtures in an ice-H_2O bath for 5 minutes. Synthesis reactions were prepared by the addition of 3.0 μL of 10X synthesis buffer (nucleotide mixture, concentration proprietary), 1.0 μL of T4 DNA

Table 3: Oligonucleotides Used to Select and Sequence pYDF31-hRad9 point mutants

Mutant	Selection Primer	Sequencing Primer
S21A	Selection #1	T2
Y28F	Selection #1	T2
S43A	Selection #1	T2
S44A	Selection #2	T2
S46A	Selection #2	T2
T121A	Selection #1	F4
T167A	Selection #2	F4
T195A	Selection #1	F4
T216A	Selection #1	F4
T250A	Selection #1	F4
T264A	Selection #2	F4
S341A	Selection #1	F4

polymerase, 1.0 μL of T4 DNA ligase, and 5.0 μL of H_2O added to the 20 μL annealing reaction. Each synthesis reaction was then incubated for 2 hours at 37°C. Each synthesis reaction was inactivated at 70°C for five minutes.

After synthesis, each mutagenesis reaction was subjected to digestion with a restriction endonuclease corresponding to the selection oligonucleotide used in the mutagenesis reaction (Table 2 and 3). In those mutagenesis reactions that used selection oligonucleotide #1 and oligonucleotide #2, *Xho*I and *Not*I respectively were used to select for populations of mutant plasmids.

Selection of mutant plasmids using *Xho*I and *Not*I was done in reactions adapted for NaCl concentrations the addition of reagents as follows: *Not*I: 15 U *Not*I, 1X BSA, and 0.75 X NEB #3; *Xho*I: 15 U *Xho*I, 1X BSA, 0.25 X NEB #2. Digestions were incubated at 37°C for 2 hours, transformed into BMH 71-18 electrocompetent bacteria, as described above and grown overnight in 5 mL of LB-amp. The following day bacterial cultures were harvested and plasmid DNA was purified. Purified plasmid DNA (2 μL) was then subjected to a secondary digestion with the appropriate restriction endonuclease for two hours at 37°C. After two hours, an additional 10 U of restriction endonuclease was added and reactions were allowed to incubate for an additional hour. After enzyme digestion, 10 μL of each digest was transformed into XL1-Blue chemically competent bacteria, and 100 μL was plated on LBamp overnight.

An initial estimate of mutagenic success was made by restriction digests of mutant plasmids to test for abolition of the selection site used in mutagenesis. For each mutant, colonies were picked in duplicate, grown overnight in 5 mL of

LBamp, plasmid DNA was purified, and 2 μL was digested with the appropriate restriction endonuclease in a total volume of 20 μL. Digests were separated by electrophoresis through 0.7% Agarose/TAE (40 mM Tris, 1 mM EDTA, 40 mM Acetic Acid, pH 8.0) gels containing 0.5 μg/mL ethidium bromide. If the selected *Xho*I or *Not*I site of the pYDF31 vector was destroyed, the sequence the hRad9 cDNA insert was determined to confirm the presence of the mutation.

2.2.3 Sequencing

All sequencing was done by an automated DNA sequencer by Canadian Molecular Research Services (Ottawa, ON). Table 3 indicates which oligonucleotides were used to sequence each of the hRad9 mutant constructs.

2.3 Mammalian Cell Culture and Manipulation

HeLa cells are a Human Papillomavirus transformed cervical cancer line (227, 228). The hTERT-RPE1 cells (Clontech) are human retinal epithelial cells that have been immortalized by stable expression of the human telomerase reverse transcriptase; hTERT-RPE1 possess a normal diploid karyotype and do not exhibit characteristics of a transformed cell line, other than an indefinite lifespan (229, 230).

2.2.3.1 Culture Conditions

All mammalian cells were cultured at 37°C under a humidified atmosphere of 5% CO_2. HeLa cells were grown in DMEM with high glucose (4.5 g/L D-glucose,

2 mM L-glutamine, and pyridoxine HCl, 110 mg/L sodium pyruvate and 3.7g/L sodium bicarbonate) supplemented with 10% heat-inactivated FBS. The hTERT-RPE1 cells were grown in DMEM F-12 (containing 15mM HEPES, L-glutamine, and pyridoxine HCL, and 1.2 g/L sodium bicarbonate) supplemented with 10% heat-inactivated FBS. Both media were supplemented with a 1X concentrations of antibiotic/antimycotic cocktail (10 units/mL penicillin G sodium, 10 μg/mL streptomycin sulfate, and 25 ng/mL amphotericin B). When cell populations became confluent they were washed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.4) and removed from the plate by 1 mL of trypsin solution (0.05% trypsin, 2.0 g/L EDTA, and 8.5 g/L NaCl) for 5 minutes at 37°C, a portion of the confluent population was used to seed new cultures. Confluent Hela cell populations were subcultured at a dilution of 1:10. Confluent hTERT-RPE1 populations were subcultured at a dilution of 1:6. All tissue culture reagents were purchased from Gibco Life Technologies.

2.2.3.2 Radiation and Drug Treatments

Thymidine (100 mM) used in cell synchronization, and Hydroxyurea (1 M) were purchased as solids, diluted in sterile H_2O to the indicated stock concentrations, and filter sterilized through a 0.2 μm filter. Nocodazole was purchased diluted to 2.5 mg/mL in DMSO, and Bleomycin was purchased at a concentration of 2 mg/mL in saline solution. Thymidine, hydroxyurea and nocodazole were all purchased from Sigma (St. Louis, MO). Bleomycin was purchased from ICN (Montreal, QC).

Cell cultures were treated with ionizing radiation using an Atomic Energy of Canada (Mississauga, ON) γ-irradiator. Ionizing radiation was delivered from a ^{137}Cs source with a dose rate of 0.76 Gy/minute. Hydroxyurea, a competitive inhibitor of ribonucleotide reductase (55), was added to tissue culture media at a concentration 10 mM for 18 hours to arrest cells in S-phase. Bleomycin, a radiomimetic drug that generates DNA double strand breaks (231), was added to tissue culture media at a concentration of 20 μg/mL for 2 hours.

2.2.3.3 Cell Synchronization

Synchronous populations of HeLa cells were derived from the double thymidine block method, the method demonstrated to yield the most synchronous populations of HeLa cells (232). Thymidine treatment yields synchronous populations of HeLa cells due to feedback inhibition; the treatment of cells with an overabundance of thymidine results in an arrest of the production of all deoxynucleotides and arrest of cells early in S-phase (232).

HeLa cell cultures that were approximately 20-30% confluent were treated with 2 mM thymidine for 18 hours, cultured in media lacking thymidine for 8 hours and were treated a second time with 2 mM thymidine for 18 hours. After double thymidine block, populations of HeLa cells synchronized to early S-phase were released from thymidine containing media to various points in the cell cycle. To generate mitotic populations of cells the microtubule poison nocodazole was added to tissue culture medium to a final concentration of 0.5 μg/mL. Nocodazole was added to cells immediately after release from thymidine

synchronization, and left for approximately 11 hours; alternatively cells were treated with nocodazole, without prior thymidine synchronization, for 18 hours.

The hTERT-RPE1 cells were synchronized by a single 24-hour treatment in 5 mM thymidine. After treatment, cells were washed in PBS, grown in media lacking thymidine, and allowed to cycle to various points of the cell cycle. This protocol was originally described for hTERT-RPE1 Cells by St.Onge *et al.* (193), and was originally developed by Minwoo Park. The degree of synchronization and cell cycle position was assayed by flow cytometry (see section 2.8).

2.2.3.4 Transfection

The *hRad9* cDNA was expressed in the pYDF31 mammalian expression vector that had its FLAG epitope cDNA excised by Kathy Kennedy. The *hRad9* cDNA was originally subcloned into the *Xho*I and *Xba*I restriction sites by Robert St.Onge. Transcription in the pYDF31- *hRad9* construct is driven by the strong constitutive SR α-promoter composed of the SV40 early promoter and a portion of the long terminal repeat of the human T-Cell leukemia virus (233).

Cells were transfected with pYDF31-hRad9 constructs using a cationic liposomal transfection reagent, as follows: HeLa cells that were approximately 40% confluent were transfected in 10 cm tissue culture dishes using 20 μL of a 2:1 molar ratio of DOPE (1,2dioleoyl-*sn*-glycerophosphatidylethanolamine) and DDAB (dimethyldioctadecylammonium bromide). Both chemicals were purchased from Sigma. The transfection reagent was mixed with 2 μg of plasmid DNA in 3.3 mL of serum free DMEM, and applied to cells for 5 hours.

After 5 hours, the transfection mixture was replaced with complete DMEM for 48 hours prior to harvest.

2.4 Cell Lysis

In all cases, cells were washed once with PBS directly on the plate, harvested by scraping with a rubber policeman in 5 mL of PBS, and collected by centrifugation in a tabletop centrifuge at 75 g for 5 minutes. For immunoblotting, cells were lysed directly in equal volumes of SDS-PAGE sample buffer within a given experiment.

In preparation for gel filtration chromatography cells were lysed by the addition of 1.5 mL of NETN lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% NP-40, 1mM EDTA, 10mM β-GP, 1mM Na_3VO_4, 1mM PMSF, 20 μg/mL aprotinin, 4 μg/mL leupeptin, and 1 μg/mL pepstatin), followed by incubation on ice for 10 minutes. Subsequently, genomic DNA was sheared ten times with a 23-gauge needle. Lysates were cleared by centrifugation in a microfuge (Eppendorf 5415 D) at 16200g for 10 minutes at 4°C.

In preparation for immunoprecipitation, cells were lysed in NETN buffer, as described above, except that NP-40 was replaced by 1% Triton X-100.

2.5 Gel Filtration Chromatography

Gel filtration chromatography was carried out using a Biologic Low Pressure Chromatography Pump System (Bio-Rad, Hercules CA). Sephacryl S-300 High Resolution size exclusion media was used to separate protein mixtures

(Bio-Rad, fractionation range of 10kDa to 1.5MDa). The gel filtration column had a final packed bed of 73cm in height and 1.5 cm in diameter. Purified proteins (670 kDa bovine thyroglobulin, 158 kDa bovine γ-globulin, and 44kDa chicken ovalbumin) used in externally standardizing the gel filtration column were all purchased from Sigma.

The gel filtration column was packed and equilibrated in PBS supplemented with the phosphatase inhibitors 50 mM β-GP and 50mM NaF to prevent hRad9 dephosphorylation; hereafter this buffer will be referred to as elution buffer. Elution buffer was filtered through a 0.2 μm filter to remove insoluble material. Initially, 5 mg each of thyroglobulin, γ-globulin, and ovalbumin were dissolved in 1.5 mL of elution buffer and fractionated over the gel filtration column. The elution of these three proteins was determined by UV absorbance at 254 nm, and served to standardize the column with species of known molecular weight for a given elution volume.

For each gel filtration chromatography experiment 11 dishes (10 cm diameter) of HeLa cells that were 20-30% confluent were synchronized by double thymidine block were released to the indicated cell cycle positions. One plate was fixed and analyzed by flow cytometry for cell cycle position. The remaining 10 plates were lysed in 1.5 mL of NETN buffer. Cleared cell lysates were loaded onto the gel filtration column and fractionated at a flow rate of 0.25 mL per minute, with total protein elution being followed by UV absorbance at 254 nm. Fractions (1 mL) were collected using an automated fraction collector (Bio-Rad). Collection was initiated once protein began to elute from the column and

fractions were collected for the duration of protein elution. To each fraction, 300 μL of 3 X SDS-PAGE sample buffer was added and fractions were boiled for 5 minutes at 100°C. Fractions were then analyzed by antibodies directed against hRad9.

2.6 Cell Fractionation and Immunoprecipitation

For the hRad9 extractability experiments described (section 3.4), 2 plates of HeLa cells synchronized by double thymidine block, treated with drugs as indicated, were then fractionated by a protocol essentially identical as described by Burtelow *et al.* (195). Cells that were washed in PBS and harvested by scraping were permeabilized in cold low salt buffer (LS buffer) (10 mM HEPES pH 7.4, 10 mM KCl, 50 μg/mL digitonin, 10mM β-GP, 1mM Na_3VO_4, 20 μg/mL aprotinin, 4 μg/mL leupeptin, 0.4 mM AEBSF, and 1 μg/mL pepstatin) for 10 minutes at 4°C with gentle agitation. After ten minutes, permeabilized cells were collected by centrifugation at 1100 *g* for 5 minutes in a microfuge. After centrifugation, supernatant was saved and labeled as the LS fraction. The pellet was washed once in 5 mL of wash buffer, which was identical to LS buffer but lacking digitonin, and collected by centrifugation at 75 *g* in an IEC Centra table top centrifuge (Thermo IEC, Needham Heights, MA) for 5 minutes at 4°C. After washing the supernatant was discarded and the pellet was lysed by the addition of 1 mL of cold high salt nuclear extraction buffer (HS buffer) (1% triton X-100, 50 mM HEPES pH 7.4, 250 mM NaCl, 30 mM $Na_4P_2O_7$, 1 mM EDTA, 10 mM β-GP, 1 mM Na_3VO_4, 20 μg/mL aprotinin, 4 μg/mL leupeptin, 0.4 mM AEBSF, and 1

μg/mL pepstatin). Samples were incubated for 10 minutes at 4°C with gentle agitation. After lysis in HS buffer, samples were cleared by centrifugation at 16,200 *g* in a microfuge for 10 minutes at 4°C, the supernatant was saved and referred to as the HS fraction. Saved LS and HS fractions were then pre-cleared with 20 μL of agarose conjugate for 30 minutes at 4°C and then cleared by briefly centrifuging at 16,200 *g* in a microfuge. Pre-cleared lysates were immunoprecipitated with 30 μL antibodies directed against hRad9 and 20 μL of agarose conjugate overnight at 4°C. The following day, immunoprecipates were harvested by centrifugation at 400g in a microfuge for 5 minutes at 4°C, washed three times with 1 mL of PBS, boiled for 5 minutes at 100°C in 50 μL of 1.5 X SDS-PAGE sample buffer, and examined by immunoblotting.

2.2.6.1 Phosphatase Assay

For phosphatase assays, soluble cell lysates were derived from two plates of HeLa cells that were harvested and lysed in 1 mL of the HS buffer used in the cell fractionation assay to ensure complete extraction of all hRad9 species from cells. Cell lysates were pre-cleared and immunoprecipitated as described for the hRad9 extractability experiments. Immunoprecipitates were washed 4 times in 1 mL of PBS of ensure complete removal of phosphatase inhibitors from hRad9 immunoprecipitates, and equilibrated in 1 X CIP reaction buffer (Boehringer Mannheim, Laval QC). Beads with bound hRad9 immunoprecipitates were then incubated at 100°C for five minutes in 60 μL of 1 X CIP reaction buffer with 1 % SDS added to elute bound hRad9 proteins from the beads. Boiled samples

were centrifuged briefly at 16,200 *g* in a microfuge, and the supernatant was divided into three for each timepoint. For each of the three aliquots within a given timepoint, samples were either diluted with 140 μL of 1 X CIP reaction buffer, 140 μL of 1 X CIP reaction buffer containing 30 units of CIP (Boehringer Mannheim), or 140 μL of 1 X CIP reaction buffer containing 30 units of CIP and made to 50 mM β-GP. Reactions were incubated at 37°C for 40 minutes and reactions were quenched by the addition of 100 μL of 3X SDS-PAGE sample buffer. Reactions were examined by immunoblotting.

2.7 SDS-PAGE and Immunoblotting

SDS-PAGE was done as described by Laemmli *et al.* (234). In all cases equivalent cell volumes of cell lysate or immunoprecipitation were separated through the indicated acrylamide concentration. After electrophoresis, proteins were transferred to 0.2 μm pure hybond nitrocellulose (Amersham-Pharmacia, Piscataway NJ) by a Trans-Blot SD semi-dry transfer apparatus (Bio-Rad), and subsequently blocked in PBS with 5 % nonfat milk powder and 0.1 % Tween-20 (Bio-Shop, Mississauga ON) for 30 minutes at room temperature. Blocked membranes were then washed once with PBS and incubated overnight with antibodies directed against hRad9 diluted in PBS at 50 ng/mL, for one hour at room temperature, or overnight at 4°C. After incubation, blots were washed twice for 10 minutes in PBS + 0.1% Tween-20 (PBST), and twice for five minutes in PBST. Blots were then incubated with goat-α-chicken HRP conjugate diluted 1 in 50,000 in PBS for one hour at 4°C, as washed as before. The hRad9

species were visualized by enhanced chemiluminescence (Perkin Elmer/NEN, Boston, MA).

The antibody directed against hRad9 described was affinity purified against recombinant hRad9, was produced by RCH antibodies, and originally described by St. Onge *et al.* (186). Secondary antibodies used for immunoblotting and flow cytometry were purchased from Jackson Immuno-Research Laboratories. The α-chicken-IgY agarose conjugate used for immunoprecipitation was purchased from Promega.

2.8 Flow Cytometry

Samples that were fixed and stained (as indicated below), were analyzed on a Beckman/Coulter EPICS Elite flow cytometer or on a Beckman/Coulter EPICS Altra flow cytometer by Derek Schultze. Within a given experiment equal numbers of events were counted for each sample, ranging between 8,000 and 20,000 events per sample.

2.2.8.1 PI Staining of Nuclei

Harvested cells were resuspended in 1 mL of PBS +1 % FBS and 1 mL of 100 % ethanol, mixed gently and incubated on ice for at least 30 minutes to fix cells. Cells were then washed twice with 5 mL of cold PBS, collected by centrifugation, and resuspended in PBS +1 % FBS + 0.5 mg/mL Ribonuclease A (RNaseA) and incubated at 37°C for 40 minutes. Cells were collected by centrifugation and

resuspended in 1 mL of PBS + 50 μg/mL propidium iodide and 0.1 mg/mL RNaseA.

2.2.8.2 Dual hRad9 Fluorescent and PI Staining

Harvested and washed cells were fixed in 1 mL of 2 % paraformaldhyde for 15 minutes at room temperature. Cells were then washed twice with PBS and incubated with 1 mL of ice cold blocking solution (1% normal goat serum, 0.1% triton X-100 in PBS) for 15 minutes on ice. Blocked cells were then treated with 1 mL of RNaseA solution (diluted to 0.5 mg/mL in blocking solution) for 30 minutes at 37°C. Cells were then collected and washed twice in 2 mL of washing solution (0.1 % Triton X-100 in PBS) and incubated with 200 μL of antibody solution (hRad9 antibody diluted to 125 ng/mL in blocking solution) for 1 hour at room temperature with gentle agitation. After primary antibody incubation, cells were washed twice with washing solution and incubated with 200 μL of Alexa-488 goat-α-chicken-IgY antibody (Jackson Immuno-research Laboratories, West Grove PA) diluted to 2 μg/mL in blocking solution for 45 minutes at room temperature with gentle agitation. Alexa-488 antibody incubations were done in darkness to avoid photo-bleaching of the fluorescent tag. After secondary antibody incubation cells were washed twice in 1 mL of washing solution and suspended in washing solution containing 50 μg/ml propidium iodide.

Chapter 3

Results

Some of the results in this section have been published previously. The data describing the cell cycle specific phosphorylation of hRad9 in HeLa and hTERT-RPE1 cells was described by St.Onge *et al.* (193).

3.1 hRad9 Exists in 3 Distinct Complexes

Genetic analyses in *S. pombe* and cross species complementation have demonstrated that hRad9, like its orthologous counterpart in fission yeast, has a role in arresting the cell cycle at G2 in response to DNA damage or replication stress (21, 126). Furthermore, the large number of reported hRad9 interacting proteins (183, 186, 187, 190, 191, 194) suggests that hRad9 may exist in a series of distinct complexes that are dynamic not only in response to DNA damage, but also within the context of the cell cycle. To address this question, hRad9 containing complexes were analyzed at various stages of the cell cycle by gel filtration chromatography. HeLa cells were subjected to a double thymidine block to yield populations of cells synchronized to early S-phase of the cell cycle; cells were then released from double thymidine block and allowed to cycle to various points in the cell cycle. Synchronous cell populations were harvested, lysed, fractionated by gel filtration chromatography, and column fractions were probed by antibodies directed against hRad9, as described in Materials and Methods. It was observed that hRad9 exists in three distinct complexes, one of which elutes with a peak at approximately 100 kDa which appears to be primarily

in the α-phosphorylation state as described by St.Onge *et al.* (193). Two others with peaks eluting at approximately 500, and 670 kDa which appeared to be in the β- and α-phosphorylation states, respectively (Figure 2A). The hRad9 containing complexes did not change in relative abundance, on the basis of spot densitometry comparing the relative signal of the three hRad9 containing complexes within a given timepoint. In addition, no new hRad9 complexes appeared at any point in the cell cycle.

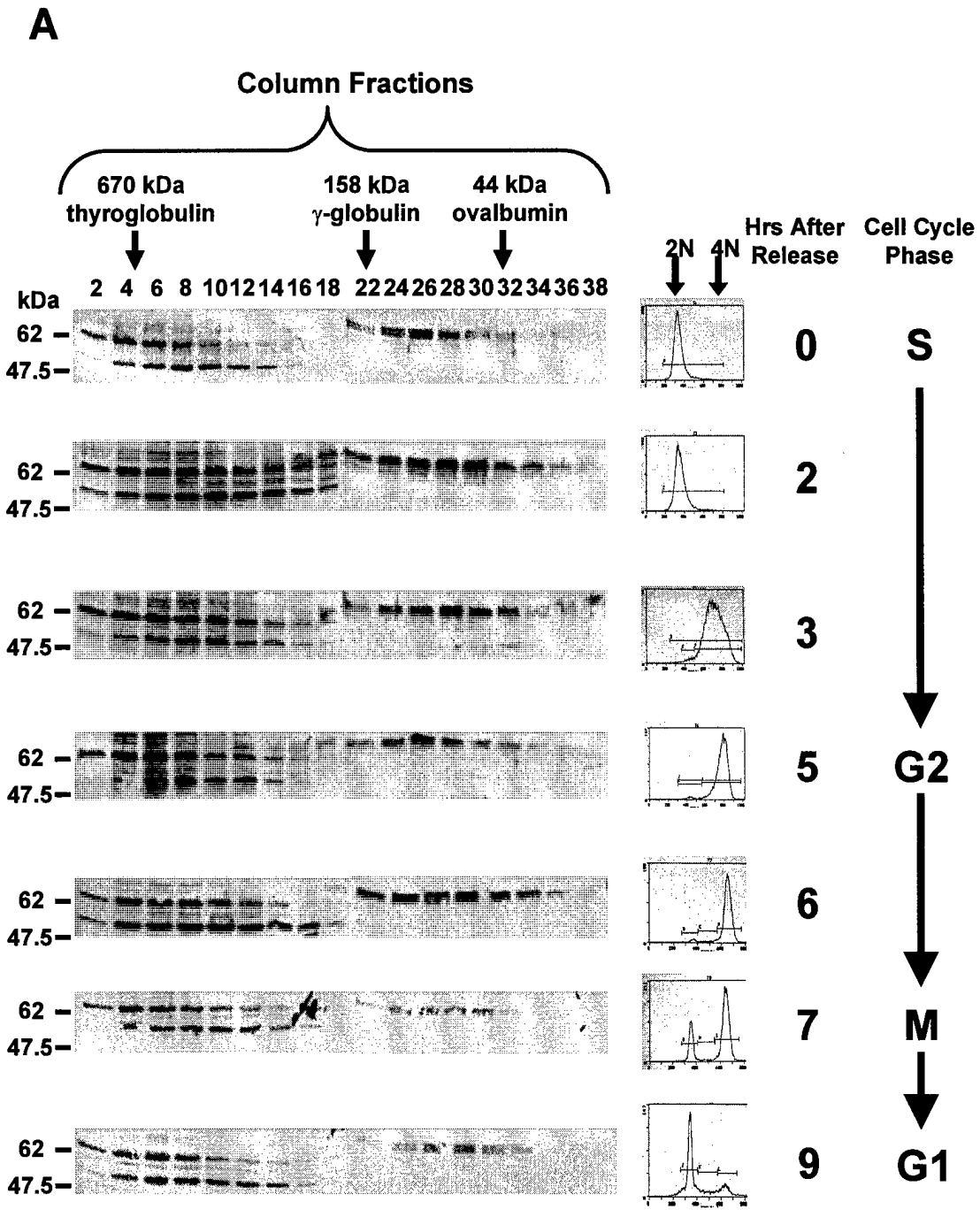
3.2 hRad9 is Phosphorylated in a Cell-Cycle Specific Manner

Upon close examination of the individual banding patterns of the hRad9 complexes visualized by gel filtration, it appeared that hRad9 eluting in the 100 kDa complex migrated as a doublet, on SDS-PAGE, at some points in the cell cycle. The presence of the hRad9 doublet in the 90 kDa complex was more clearly demonstrated by the comparison of identical fractions from each gel filtration timepoint on a single acrylamide gel that was allowed to run further (Figure 2B). This result suggested that hRad9 was undergoing a cell-cycle specific modification in Hela cells during G2/M.

3.2.1 G2/M Specific hRad9 Phosphorylation in HeLa Cells

In order to determine if the slower migrating hRad9 species observed by SDS-PAGE at G2/M were a significant fraction of the total hRad9 in the cell, hRad9 was compared in lysates from synchronized cell populations by antibodies

Figure 2. hRad9 elutes from a gel filtration column in 3 distinct complexes. **A,** HeLa cell lysates synchronized in early S Phase by double thymidine block were released and released to the indicated times. Synchronized cell lysates were then fractionated over a Sephacryl S-300 gel filtration column, standardized as indicated, and collected in 1 mL fractions. A portion of cells from each timepoint were analyzed by flow cytometry to determine cell cycle phase. Fractions were separated by SDS-PAGE and immunoblotted with antibodies directed against hRad9. **B,** Comparison of hRad9 in fraction 26 from different cell cycle positions by αhRad9 immunoblotting.


A
Column Fractions
670 kDa thyroglobulin
158 kDa γ-globulin
44 kDa ovalbumin
2 4 6 8 10 12 14 16 18 22 24 26 28 30 32 34 36 38
kDa
62
47.5
2N 4N
Hrs After Release
Cell Cycle Phase
0 S
2
3
5 G2
6
7 M
9 G1
B
Fraction 26
kDa
0 2 5 6 9
Hours After Release
62

directed against hRad9 (Figure 3). Consistent with the gel filtration result, hRad9 undergoes a modification specific to G2/M of the cell cycle, which does not persist into G1 (Figure 3).

Given that hRad9 is extensively phosphorylated, and that hRad9 is proposed to have a role in controlling the G2/M transition, the likelihood of hRad9 being phosphorylated in a cell cycle dependent manner was high. In order to test whether the G2/M specific mobility shift of hRad9 was a phosphorylation dependent phenomenon, hRad9 derived from cells synchronized to S or G/M phase of the cell cycle was treated with CIP and analyzed by antibodies directed against hRad9. Cells were harvested, lysed, and the hRad9 from equivalent amounts of cell lysate was immunoprecipitated. Immunoprecipitates were treated with CIP, as indicated, in either the presence or absence of the phosphatase inhibitor β-GP. Samples were then analyzed by immunoblotting with antibodies against hRad9. It was observed that upon CIP treatment the slower migrating hRad9 species present in G2/M phase HeLa cells were abolished, but persisted in the presence of phosphatase inhibitor (Figure 4), indicating that the cell-cycle specific forms of hRad9 are the result of a phosphorylation. These cell cycle specific hRad9 forms will collectively by referred to as hRad9σ.

3.2.2 Cell Cycle Specific hRad9 Phosphorylation is Independent and Distinct from that Induced by Ionizing Radiation

Figure 3. HeLa cells during G2 and M phases of the cell cycle exhibit Novel slow-migrating forms of hRad9. HeLa cells were synchronized by double thymidine block and harvested at the indicated cell cycle phases. Cells were lysed and subjected to immunoblot analysis with antibodies directed against hRad9 (top) or stained with PI and analyzed for DNA content/cell cycle phase by flow cytometry (bottom).

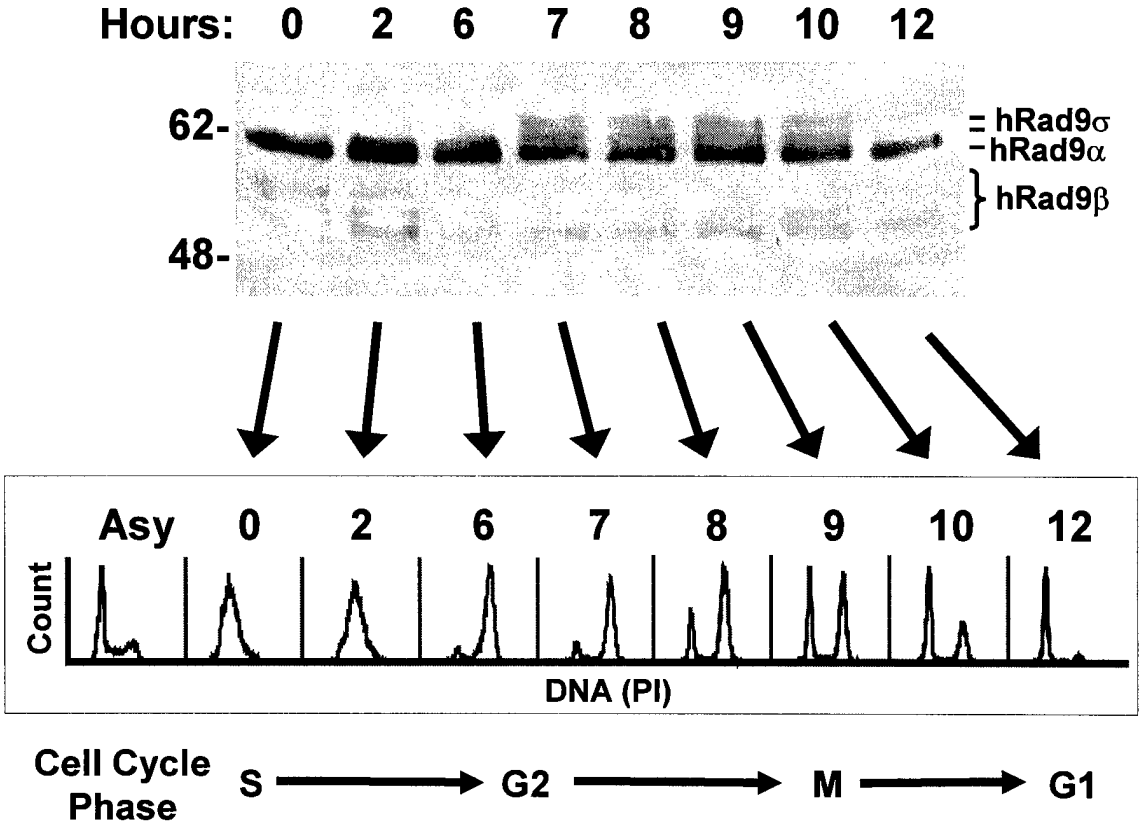
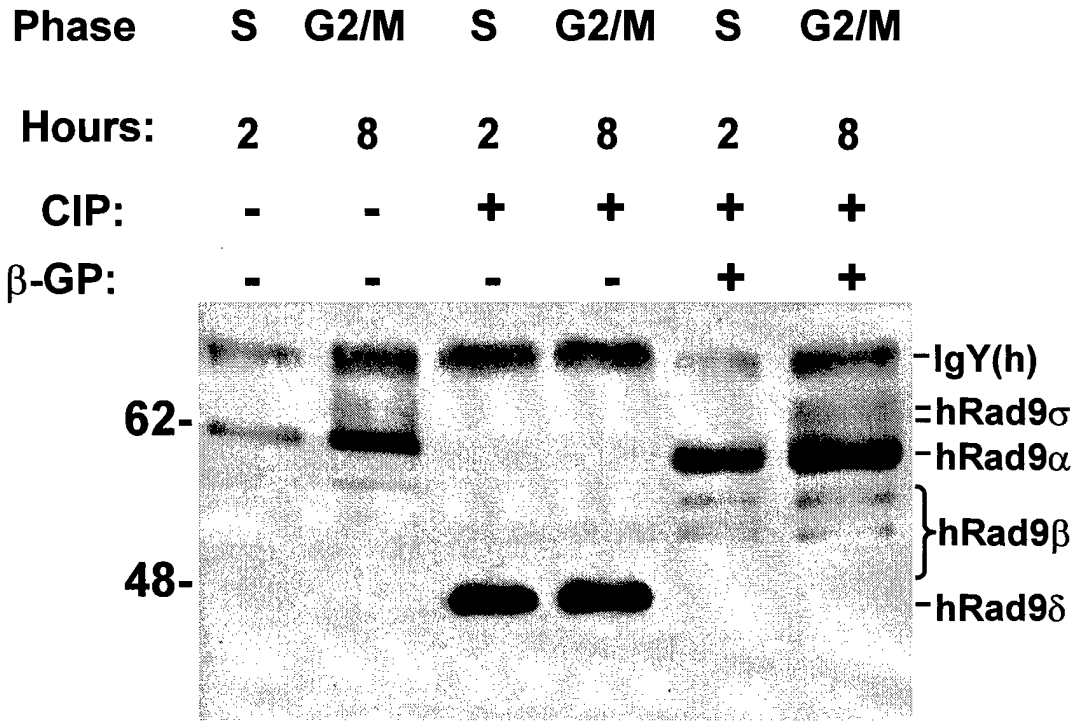

Hours: 0 2 6 7 8 9 10 12
62-
48-
hRad9σ
hRad9α
hRad9β
Asy 0 2 6 7 8 9 10 12
Count
DNA (PI)
Cell Cycle Phase
S
G2
M
G1

Figure 4. hRad9 is phosphorylated in a cell cycle dependent manner. Antibodies directed against hRad9 were used to immunoprecipitate hRad9 from HeLa cells synchronized to G2/M and S-phase of the cell cycle. Immunoprecipitates were then incubated in the presence of 40 units of CIP and the phosphatase inhibitor β-GP, as indicated. Proteins were separated by 10% SDS-PAGE and immunoblotted with antibodies directed against hRad9.

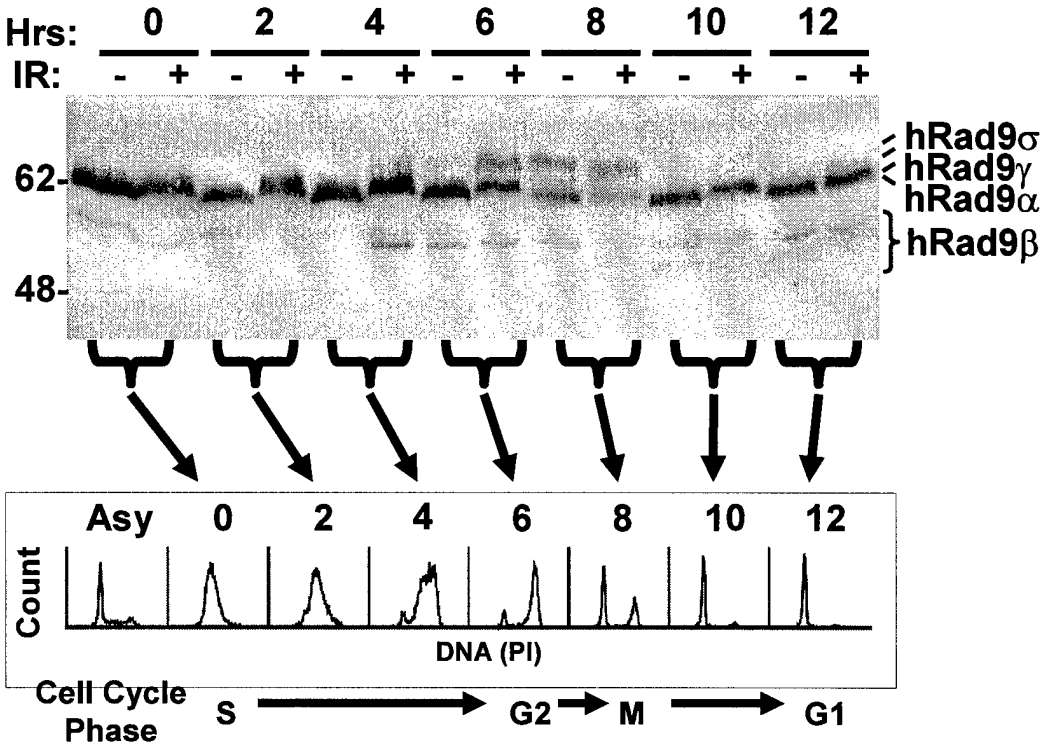

Phase S G2/M S G2/M S G2/M
Hours: 2 8 2 8 2 8
CIP: - - + + + +
β-GP: - - - - + +
62-
48-
IgY(h)
hRad9σ
hRad9α
hRad9β
hRad9δ

Since the ATM kinase has been demonstrated to phosphorylate hRad9 in response to DNA damage (146, 193), we examined whether the G2/M specific hRad9 phosphorylation is dependent upon the ATM induced phosphorylation. Synchronized HeLa cell lysates were examined at various points in the cell cycle, and were treated with 20 Gy of IR 30 minutes prior to harvest, and examined by immunoblotting (Figure 5). An IR-dependent mobility shift of the hRad9α form by SDS-PAGE was observed at all points in the cell cycle. We have termed this hRad9 form hRad9γ, and we and others have demonstrated this mobility shift to be a result of phosphorylation of hRad9 by the ATM kinase (146, 193) (Figure 5). It was also observed that the formation of hRad9σ occurred even when hRad9γ was not present, but hRad9σ formation was enriched upon treatment with ionizing radiation (Figure 5). Furthermore, work in our lab has demonstrated that hRad9σ forms still form in hRad9 point mutants that cannot be phosphorylated by ATM or ATR (193).

3.2.3 Cell Cycle Specific Inducible hRad9 Phosphorylation in hTERT-RPE1 Cells

Because the initial observation of hRad9σ was made in HeLa cell lysates, it was important to ascertain if the G2/M specific hRad9 phosphorylation was unique to the highly transformed phenotype, and aneuploid genotype of Hela cells, or whether in fact hRad9σ can be detected in healthy epithelial cells with a diploid karyotype. Cell cycle specific hRad9 phosphorylation was examined in karyotypically normal hTERT-RPE1 epithelial (hTERT) cells. Synchronous populations of hTERT cells were achieved by a single 24-hour thymidine block.

Figure 5. Phosphorylation of hRad9 at G2/M is enhanced by IR, but is distinct from IR induced phosphorylation that occurs at all points of the cell cycle. Synchronized HeLa cells, released to the indicated times, were lysed in SDS-PAGE sample buffer and probed by immunoblotting with antibodies directed against hRad9 (top), or followed by flow cytometry (bottom). At each timepoint cells were treated with 0 or 20 Gy of ionizing radiation 30 minutes prior to harvest, as indicated.


Hrs:
0
2
4
6
8
10
12
IR:
- + - + - + - + - + - + - +
62-
48-
hRad9σ
hRad9γ
hRad9α
hRad9β
Asy
0
2
4
6
8
10
12
Count
DNA (PI)
Cell Cycle Phase
S
G2
M
G1

Cell populations were released and treated with either 20 Gy or 0 Gy of IR, 30 minutes prior to harvest, at all points in the cell cycle. After harvest, cell lysates were examined by antibodies directed against hRad9 (Figure 6). Consistent with observations in HeLa cells, hRad9σ is only present in cells at G2/M of the cell cycle. However, hRad9σ was not present in undamaged epithelial cells at G2/M; hRad9σ forms are dependent on cell cycle position and the presence of exogenous DNA damage in a karyotypically normal cell line (Figure 6).

3.3 hRad9 Mutagenesis

In an attempt to determine specific sites of hRad9σ phosphorylation serine, threonine, and tyrosine residues of hRad9 that are conserved between the murine, human, and fission yeast orthologs, as well as residues which demonstrated a high probability of being phosphorylated by phosphorylation site prediction programs (235), were targeted by site directed mutagenesis (Figure 7). Twelve hRad9 single point mutants were generated and compared for their effects on hRad9 phosphorylation patterns and for G2 checkpoint defects.

3.3.1 Targeted Disruption of Conserved Residues does not Abrogate G2/M Phosphorylation

In order to determine if any single hRad9 point mutants altered or abolished the formation of hRad9σ, mutant constructs were transfected into HeLa cells, and 18 hours prior to harvest were treated with the microtubule poison nocodazole (0.5 μg/mL). Treatment with nocodazole arrests cell cycle progression at

Figure 6. hRad9 phosphorylation at G2/M is inducible by IR in hTERT-RPE1 cells. hTERT-RPE1 cell populations were synchronized by a single thymidine block and released to the indicated timepoints, and treated with 0 or 20 Gy of ionizing radiation 30 minutes prior to harvest. Cells were then either lysed in 1.5 X SDS-PAGE sample buffer and analyzed by immunoblotting with antibodies directed against hRad9 (top), or stained with PI and analyzed by flow cytometry.

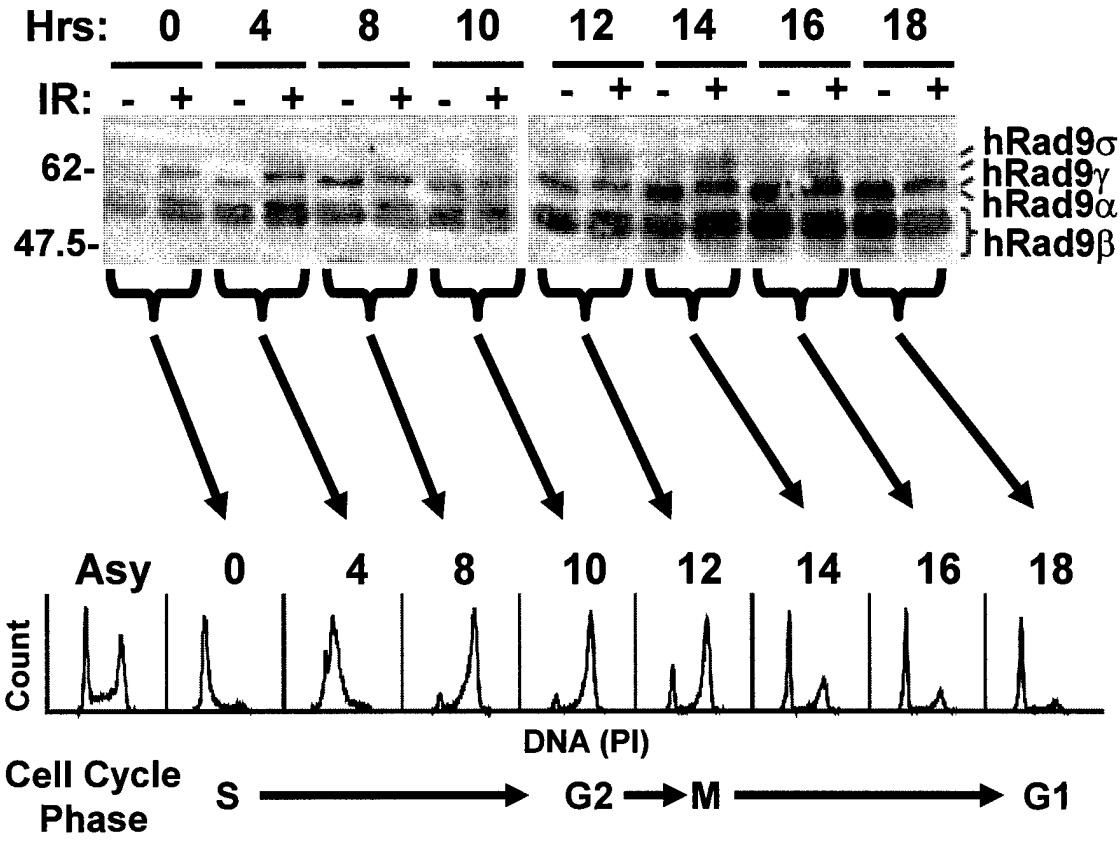
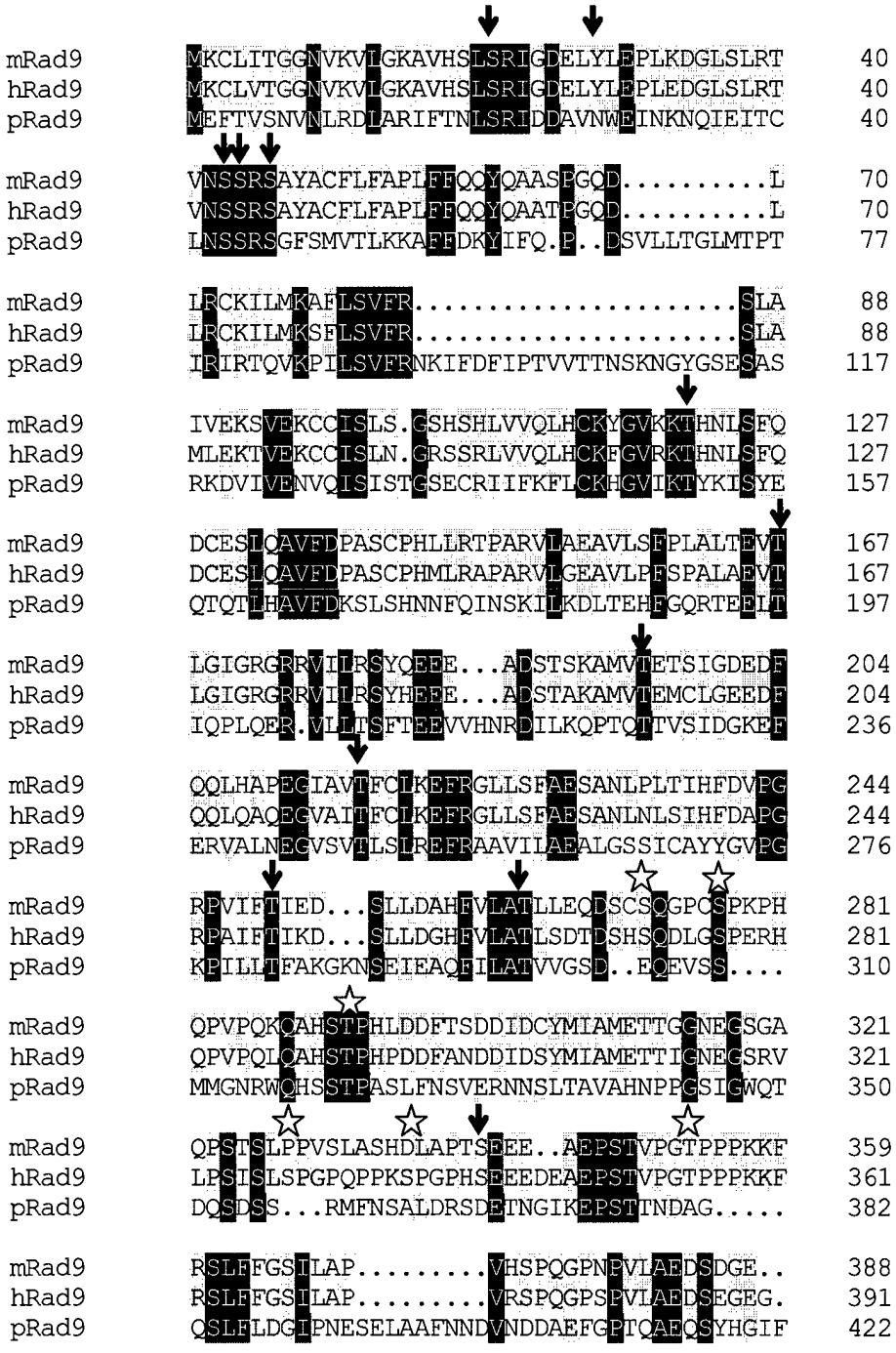

Hrs: 0 4 8 10 12 14 16 18
IR: - + - + - + - + - + - + - + - +
62-
47.5-
hRad9σ
hRad9γ
hRad9α
hRad9β
Asy 0 4 8 10 12 14 16 18
Count
DNA (PI)
Cell Cycle Phase
S
G2
M
G1

Figure 7. Primary amino acid sequence alignment of human, mouse, and fission yeast hRad9 orthologs. Twelve serine, threonine, and tyrosine residues of hRad9 that were conserved between the human, murine, and *S. pombe* Rad9 orthologs, and exhibited a high score in phosphorylation site prediction programs were targeted by site directed mutagenesis to see if their elimination would abrogate hRad9 cell cycle specific phosphorylation. Arrows indicated residues targeted by site directed mutagenesis; stars denote known hRad9 phosphorylation sites.



↓ = targeted residue

☆ = known hRad9 phosphorylation site

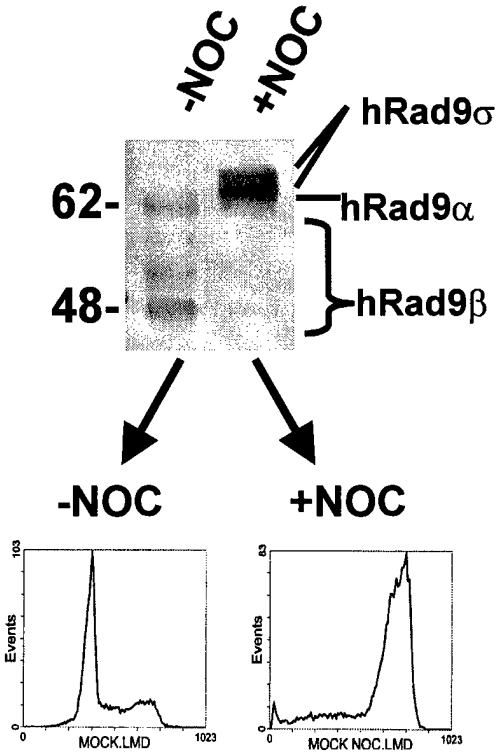
metaphase of mitosis, eliciting formation of hRad9σ, even in transiently transfected over-expressed proteins (Figure 8A) (193). Populations of treated and untreated hRad9 transfectants were compared by immunoblotting. Induction of hRad9σ in over-expressed proteins is concurrent with nocodazole treatment, and subsequent mitotic arrest (Figure 8A). Subsequently, each of the 12 mutant constructs were compared to wild-type hRad9 and mock transfected samples in the presence and absence of nocodazole (Figure 8B). None of the hRad9 point mutants showed elimination of the hRad9σ form under these conditions (Figure 8B).

3.3.2 Over-expression of Rad9 Mutants does not Effect Normal Cell Cycle Progression or Prevent G2 Arrest in Response to Ionizing Radiation.

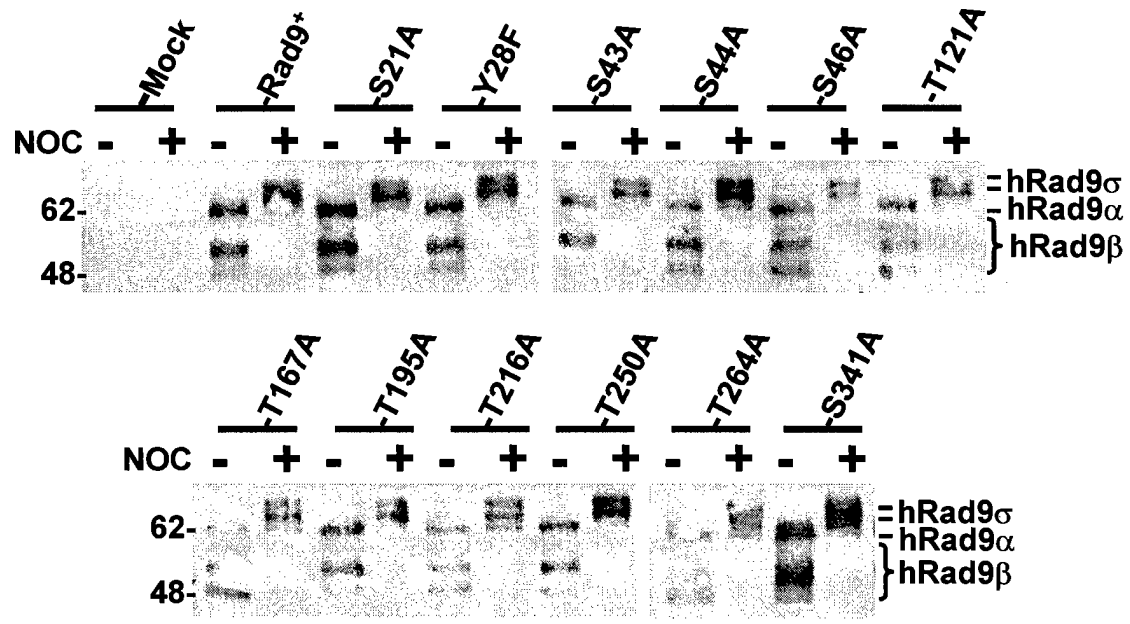
Some of the single hRad9 mutants generated (S21A, S43A, S44A, and S46A) correspond to residues in that when mutated in tandem result in increased genotoxin sensitivity and minor checkpoint defects in *S.pombe* (236). Given the possible functional implications, the 12 hRad9 point mutants were also analyzed for G2 checkpoint defects. In order to assay for G2 checkpoint activity populations of HeLa cells transfected with hRad9 point mutants were synchronized by a single 24 hour thymidine block. HeLa cell populations were then released from thymidine block and allowed to cycle for 5.5 hours, yielding a population of cells at Late-S/Early G2 of the cell cycle, cells were then treated with either 0 or 4 Gy of IR. Cells were then harvested an additional 6.5 hours

Figure 8. Use of Nocodazole for analysis of hRad9 single point mutants. A, HeLa cells were transiently transfected with wild type hRad9, and were treated with nocodazole 18 hours prior to harvest, or left untreated. Cells were either harvested in 1.5 X SDS-PAGE sample buffer or fixed and stained with PI for analysis by flow cytometry. **B,** HeLa cells were transfected with wild-type hRad9 and each of the 12 single hRad9 point mutants, and treated with nocodazole, as indicated. Cells were then harvested in and subjected to immunoblot analysis with antibodies directed against hRad9.

A


-NOC
+NOC
hRad9σ
62-
hRad9α
48-
hRad9β
-NOC
+NOC
Events
MOCK.LMD
MOCK NOC.LMD
0
1023

B


-Mock
-Rad9+
-S21A
-Y28F
-S43A
-S44A
-S46A
-T121A
NOC - + - + - + - + - + - + - + - +
62-
48-
=hRad9σ
-hRad9α
}hRad9β
-T167A
-T195A
-T216A
-T250A
-T264A
-S341A
NOC - + - + - + - + - + - +
62-
48-
=hRad9σ
-hRad9α
}hRad9β

later, 12 hours after release from thymidine block, with irradiated cells arrested at G2/M and untreated cells having cycled back into G1 of the cell cycle. Cells were then harvested, fixed, and stained by αhRad9 immunofluorescent antibodies and PI. On the basis of levels of hRad9 fluorescence, populations of transfected (high hRad9 fluorescence) and non-transfected cells (low hRad9 fluorescence) could be differentiated by flow cytometry and assayed separately for cell cycle position on the basis of PI staining, illustrated in Figure 9. Using flow cytometry, the cell cycle position of transfected cells could be compared to that of non-transfected cells, and the effect of the transfected point mutants on cell cycle position could be determined.

It was observed in non-irradiated populations of cells that by 12 hours after release from thymidine synchronization that both transfected and non-transfected cells had returned to G1 of the cell cycle. The lack of a perturbation in the progression though G2/M phase of the cell cycle indicates that overexpression of the hRad9 point mutants elicits a checkpoint dependent arrest in cell cycle progression in this assay (Figure 10). Furthermore, cells transfected with hRad9 point mutants all exhibited a checkpoint-dependent arrest at G2/M at 12 hours after release, following IR treatment 5.5 hours after release from thymidine block. The presence of G2 arrest of cells in response to IR despite transfection with the hRad9 mutants indicates that none of the hRad9 point mutants studied impaired normal IR induced G2/M checkpoint function in this assay (Figure 11).

3.4 Formation of Extraction Resistant hRad9 Complexes Not Effected by

G2/M Specific Phosphorylation.

The biochemical function of hRad9 in the cell is unclear, and thus a functional assay for hRad9 function is currently unavailable. However, hRad9 containing complexes have been shown to become resistant to extraction in a low-salt buffer, a result proposed to be a result of the 9-1-1 complex binding to chromatin in response to DNA damage (143, 195). Accordingly, the extractability of hRad9 within the context of the cell cycle was examined. Initially, the published extraction resistance of hRad9 was recapitulated in asynchronous cells. Asynchronous HeLa cells were either left untreated, or treated with DNA replication inhibitors thymidine, or hydroxyurea (HU), or DNA damaging agents bleomycin and IR. Equivalent cell volumes were harvested lysed in low salt (LS) permeabilization buffer, washed, and the remaining nuclear pellet was extracted in high salt (HS) extraction buffer. Fractionated cell lysates where then immunoprecipitated using antibodies directed against hRad9. Immunoprecipitates demonstrated that not only DNA damage stimuli, but also replication inhibition converted hRad9 to an extraction resistant form (Figure 12), as evidenced by the increase in hRad9 present in the HS fraction. The inducible increase in hRad9 content in the HS fraction is consistent with hRad9 being loaded onto chromatin in response to DNA damage, or replication inhibition. It is also noteworthy that in cells treated with hydroxyurea all of the hRad9β forms normally visible in hRad9 immunoprecipitates disappeared, similar to cells synchronized to G2/M by thymidine synchronization or treated with nocodazole.

Figure 9. Schematic diagram of analysis of hRad9 transfected versus non-transfected cells analyzed by flow cytometry. HeLa cells were synchronized by a single thymidine block and released for 5.5 hours before being treated with 0 or 4 Gy of ionizing radiation. Cells were then harvested 12 hours after release from thymidine block, fixed in paraformaldhyde. Cells were then analyzed for hRad9 content (Y-axis) or DNA content (X-axis) and analyzed by flow cytometry. Treated cell populations were then gated by flow cytometry for hRad9 fluorescence and transfectants were analyzed for DNA content by PI staining to determine cell cycle position of transfected and non-transfected populations of cells.

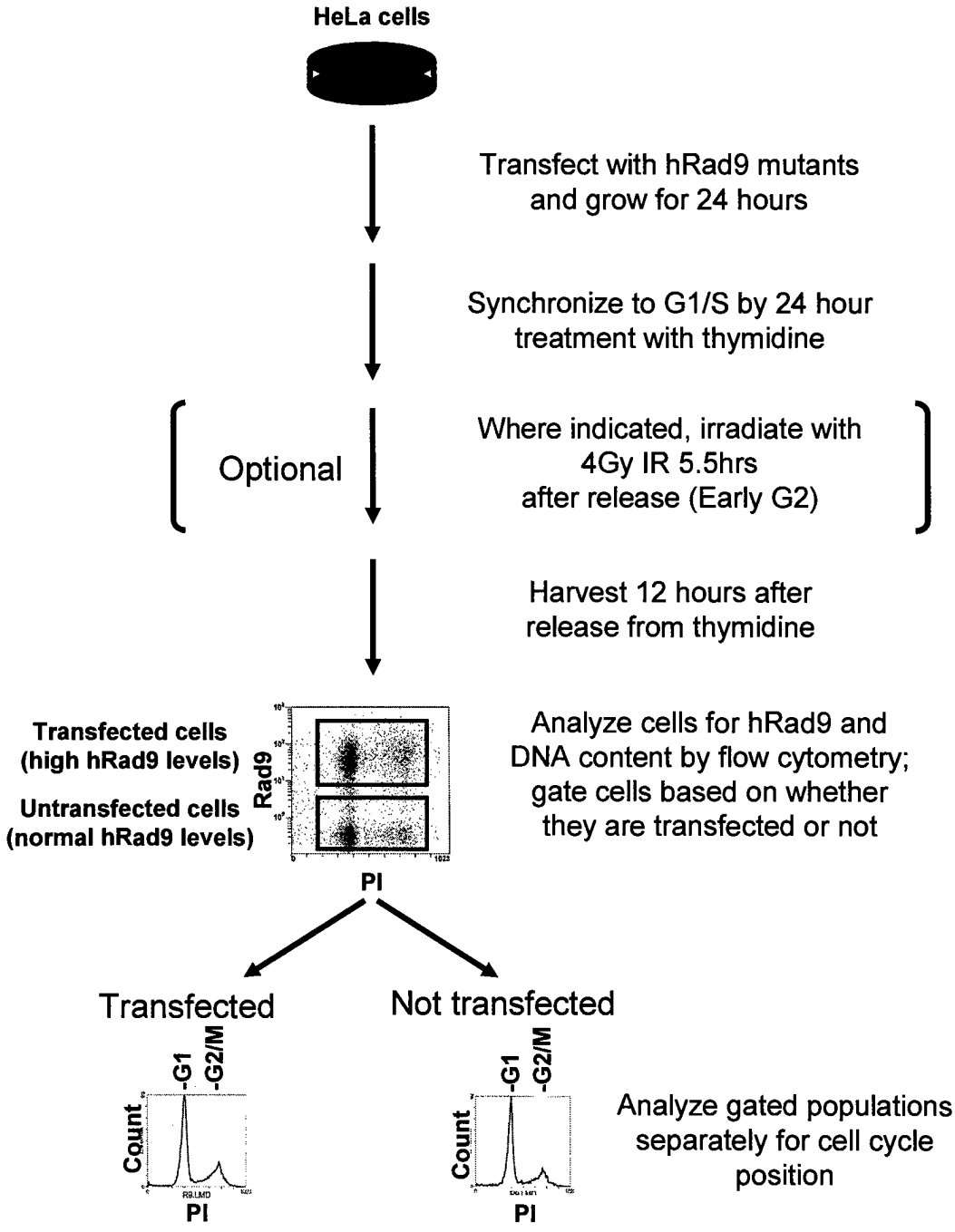

HeLa cells
Transfect with hRad9 mutants and grow for 24 hours
Synchronize to G1/S by 24 hour treatment with thymidine
Optional
Where indicated, irradiate with 4Gy IR 5.5hrs after release (Early G2)
Harvest 12 hours after release from thymidine
Transfected cells (high hRad9 levels)
Untransfected cells (normal hRad9 levels)
Rad9
PI
Analyze cells for hRad9 and DNA content by flow cytometry; gate cells based on whether they are transfected or not
Transfected
Not transfected
-G1
-G2/M
Count
PI
Analyze gated populations separately for cell cycle position

Figure 10. Overproduction of hRad9 point mutants does not inhibit normal passage through G2/M. Wild-type hRad9 and each of the 12 individual point mutants were analyzed by flow cytometry. Transfected cells were synchronized by single thymidine block for 18 hours, 24 hours after transfection. Cells were then released from thymidine block for 12 hours, harvested, and treated with fluorescent antibodies directed hRad9 and PI. Transfectants were analyzed for cell cycle position based upon DNA content.

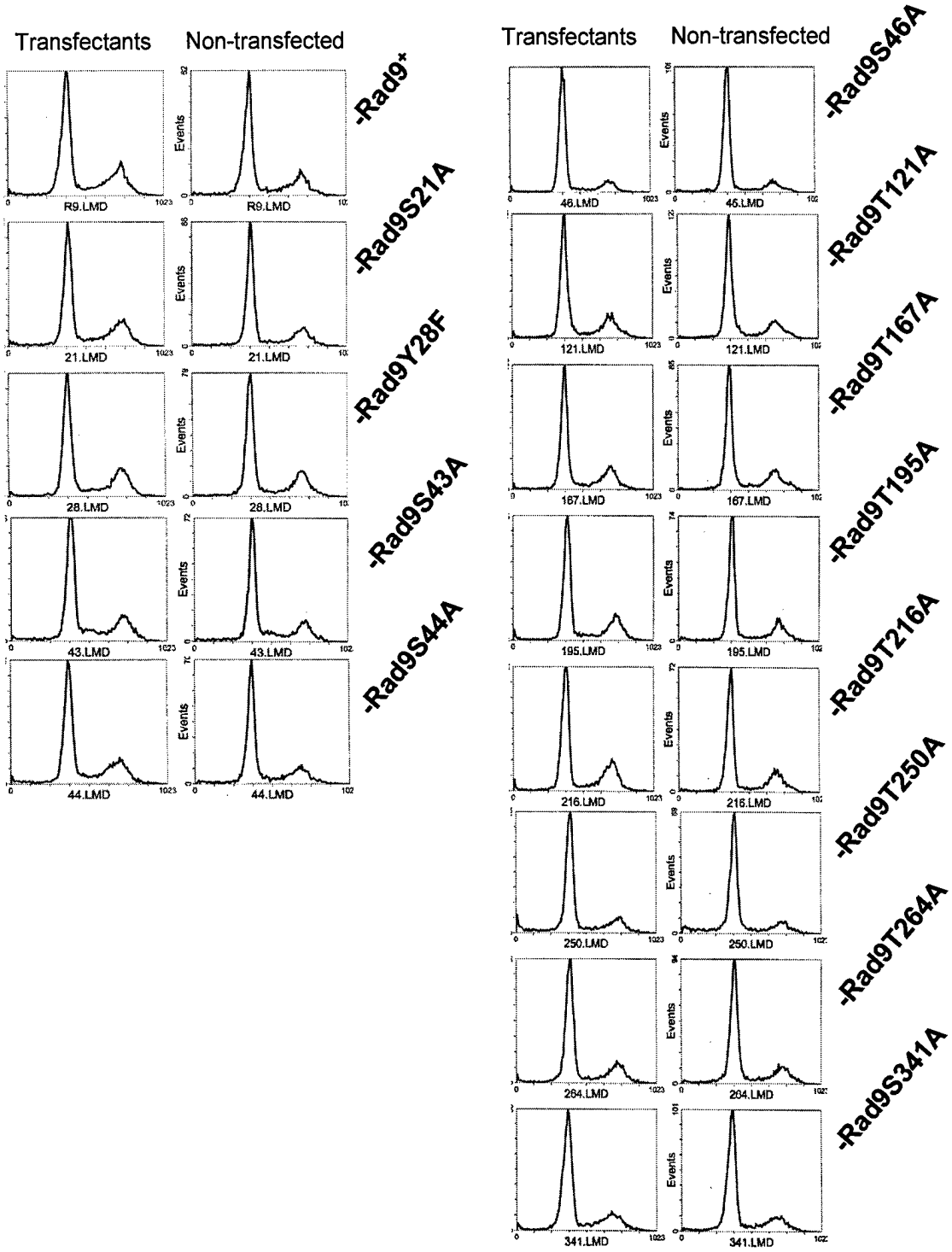

Transfectants
Non-transfected
-Rad9+
-Rad9S21A
-Rad9Y28F
-Rad9S43A
-Rad9S44A
-Rad9S46A
-Rad9T121A
-Rad9T167A
-Rad9T195A
-Rad9T216A
-Rad9T250A
-Rad9T264A
-Rad9S341A
Events
R9.LMD
21.LMD
28.LMD
43.LMD
44.LMD
46.LMD
121.LMD
167.LMD
195.LMD
216.LMD
250.LMD
264.LMD
341.LMD

Figure 11. Overproduction of hRad9 point mutants does not effect ionizing radiation induced normal G2 arrest. Wild-type hRad9 and each of the 12 hRad9 point mutants were analyzed by flow cytometry. Transfected cells were synchronized by single thymidine block for 18 hours, 24 hours after transfection. Cells were then released from thymidine block for 5.5 hours, treated with 4 Gy of IR, and harvested 12 hours after their initial release to yield populations of G2/M arrested cells. Harvested cells were treated with fluorescent antibodies directed against hRad9 and with PI. Transfected and non-transfected cells were distinguished by flow cytometry. Cells were analyzed for cell cycle position based upon DNA content.

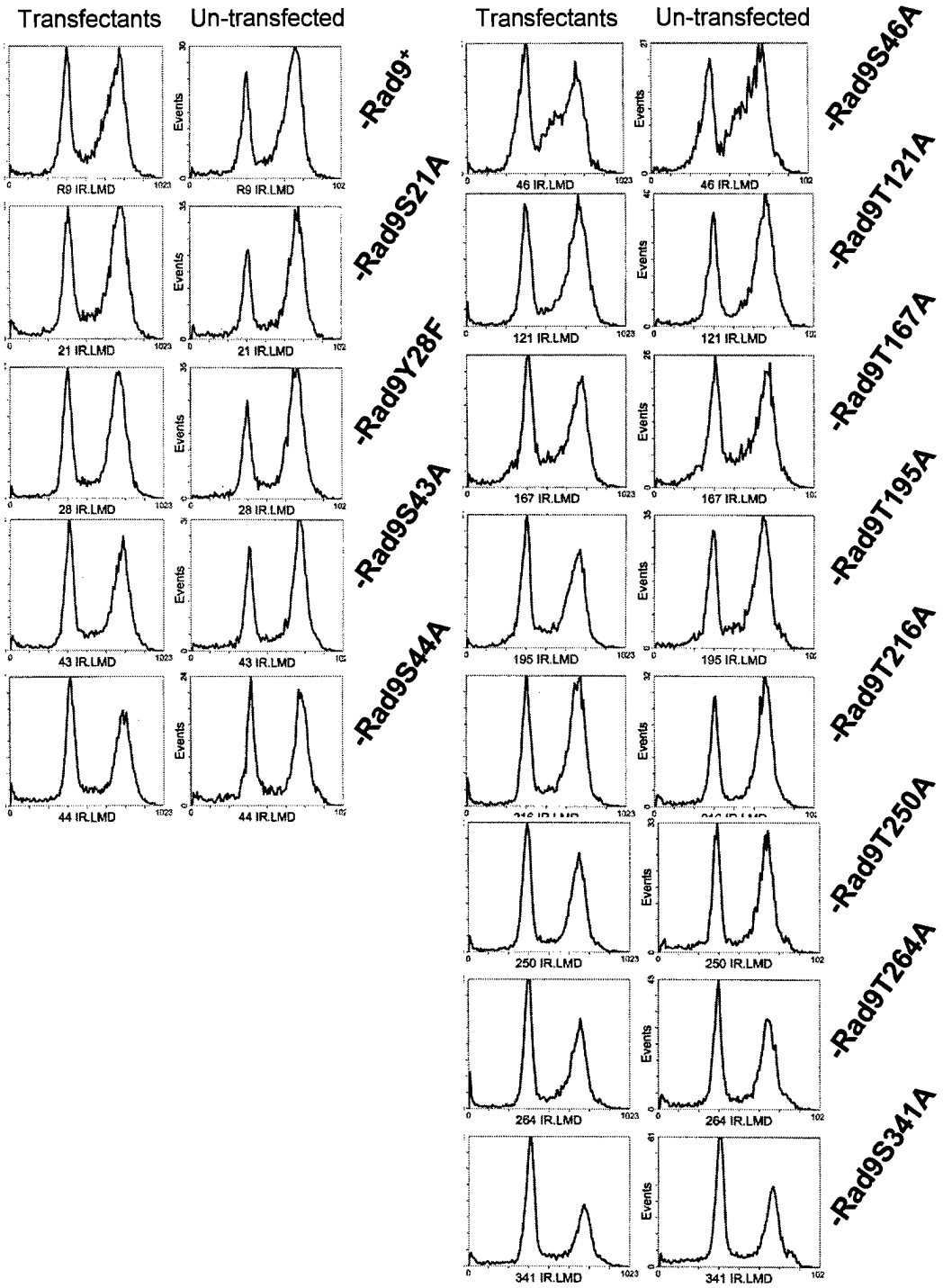
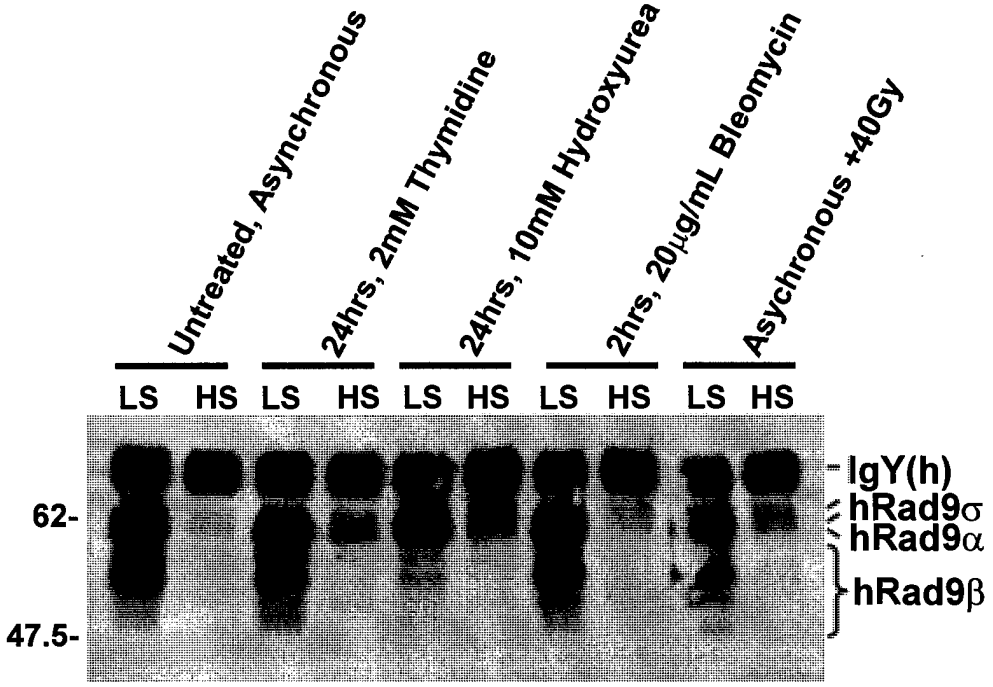
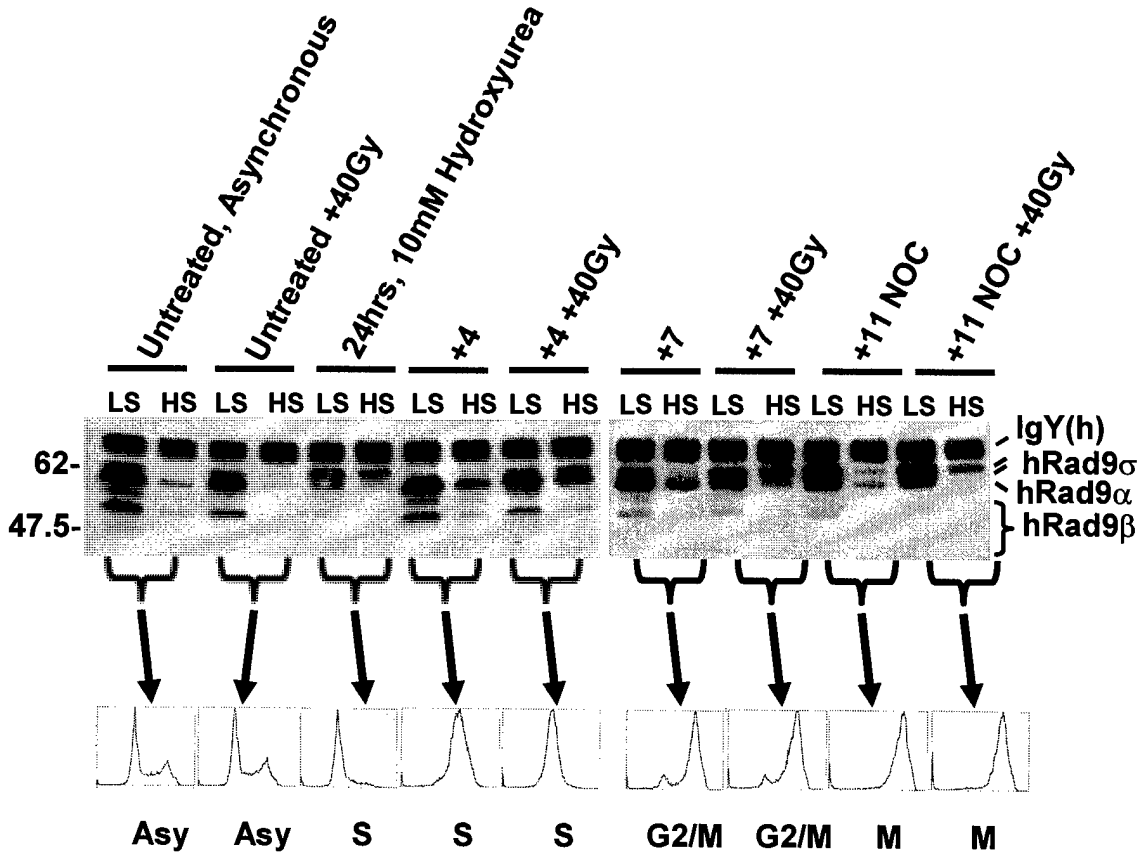

Transfectants
Un-transfected
Transfectants
Un-transfected
-Rad9+
-Rad9S21A
-Rad9Y28F
-Rad9S43A
-Rad9S44A
-Rad9S46A
-Rad9T121A
-Rad9T167A
-Rad9T195A
-Rad9T216A
-Rad9T250A
-Rad9T264A
-Rad9S341A
Events
R9 IR.LMD
21 IR.LMD
28 IR.LMD
43 IR.LMD
44 IR.LMD
46 IR.LMD
121 IR.LMD
167 IR.LMD
195 IR.LMD
250 IR.LMD
264 IR.LMD
341 IR.LMD

Figure 12. DNA replication arrest and DNA damage converts constitutively phosphorylated hRad9 to an extraction resistant form. HeLa cells were treated with the DNA replication inhibitors (thymidine and HU), DNA damaging agents (Bleomycin and IR), or left untreated, as indicated. Cells were then harvested, permeabilized, and low (LS) and high salt (HS) extracts were prepared. Human Rad9 protein was immunoprecipitated with antibodies directed hRad9, immunoprecipitates were analyzed by immunoblotting with the same antibody.


Untreated, Asynchronous
24hrs, 2mM Thymidine
24hrs, 10mM Hydroxyurea
2hrs, 20μg/mL Bleomycin
Asychronous +40Gy
LS HS LS HS LS HS LS HS LS HS
62-
47.5-
IgY(h)
hRad9σ
hRad9α
hRad9β

This effect is unique to HU treatment as cells synchronized to S-phase by thymidine block and release does not exhibit loss of hRad9β forms. The extractability of hRad9 was then examined in synchronized HeLa cells to determine if DNA damage at specific points of the cell cycle was enhanced or inhibited by the formation of hRad9σ. Cells that were either asynchronous or synchronized to S-phase, G2, or M and treated with IR or HU as indicated. In all cases, the extraction resistant form of hRad9 was enriched in cells that had been synchronized by thymidine synchronization to S, G2, and M phase of the cell cycle. Furthermore, it was observed that in all cases of ionizing radiation treated cells, synchronized or otherwise, that the extraction resistant form of hRad9 appeared to be preferentially phosphorylated in a banding pattern consistent with hRad9σ (Figure 13). In samples which had been synchronized into mitosis by double thymidine block and subsequent nocodazole treatment it was evident that a hyper-phosphorylated form of hRad9, consistent with hRad9σ was made extraction resistant in response to IR treatment (Figure 13, samples +11 NOC and +11 NOC+40Gy). The formation of extraction resistant hRad9 forms at all points in the cell cycle suggests that all characterized forms of hRad9 are competent to be loaded onto chromatin.

Figure 13. Human Rad9 is converted to extraction resistant form independent of cell cycle position or phosphorylation state. Synchronized HeLa cells were released to the indicated timepoints and either treated with 0 or 40 Gy of IR, 45 minutes prior to harvest. Cells were also treated with HU as a positive control. Cells were then harvested, permeabilized, and low (LS) and high salt (HS) extracts were prepared. hRad9 protein was immunoprecipitated with antibodies directed against hRad9, immunoprecipitates were immunoblotted hRad9 using the same antibody.


Untreated, Asynchronous
Untreated +40Gy
24hrs, 10mM Hydroxyurea
+4
+4 +40Gy
+7
+7 +40Gy
+11 NOC
+11 NOC +40Gy
LS HS LS HS LS HS LS HS LS HS LS HS LS HS LS HS LS HS
62-
47.5-
IgY(h)
hRad9σ
hRad9α
hRad9β
Asy Asy S S S G2/M G2/M M M

Chapter 4

Discussion

4.1 hRad9 Exists in 3 distinct Complexes

It was demonstrated that hRad9 exists in three distinct complexes that are invariant with respect to their relative abundance or cell cycle position. The invariant nature of the hRad9 containing complexes suggests that the function of hRad9 containing complexes is not modulated by their assembly, disassembly, or degradation. The smallest hRad9 containing complex eluting at an approximate molecular weight of 100 kDa is in agreement with the heterotrimeric 9-1-1 complex described, and is consistent with another reports stating that hRad9, hHus1, and hRad1 co-fractionate in a complex of identical size by gel filtration chromatography (181, 188). However, the two larger hRad9 containing complexes (500 and 670 kDa, approximately) observed were not consistent with the work of another group, who only observed a single hRad9 containing complex (188).

Despite the disparity in findings between the work of Burtelow *et al.* (188) and the work described here, the finding that hRad9 exists two other larger complexes is highly probable and is supported by several lines of evidence. As described in the introduction, it has been demonstrated that hRad9 interacts with a number of proteins other than hHus1 and hRad1 (183, 190-192). Furthermore, in addition to the known hRad9 interacting partners, it has been demonstrated that an uncharacterized phosphoprotein of approximately 180 kDa is present in hRad9 immunoprecipitates from ^{32}P-metabolically labeled cells (146). In

addition, given that there is not a *bona fide* function ascribed to the 9-1-1 complex, it is very possible that other factors are required to bind hRad9 in order to mediate the downstream signal transduction resulting in the inhibition of Cdc2. All of these factors suggest that hRad9, and likely hHus1 and hRad1 exist in large complexes possibly involved in genome surveillance or the mediation of G2 checkpoint activity.

Interestingly, the two larger hRad9 containing complexes are in different phosphorylation states, with the 670-kDa hRad9 containing complex being in the α-phosphorylation state and the 500-kDa complex consisting of the β-form of hRad9. It is unclear if the 500-kDa complex is representative of an hRad9 phosphorylation state that is naturally occurring in the cell, as it is very rare that the β-form of hRad9 can be visualized by western blotting. However, given that the 500-kDa complex elutes distinctly from the 670-kDa complex it is possible that by gel filtration chromatography dividing the pool of hRad9α into two parts hRad9β forms become more visible. However, it cannot be discounted that the phosphorylation state of the 500-kDa hRad9 complex is a result of dephosphorylation during fractionation over the gel filtration column, despite the use of large amounts of phosphatase inhibitors.

4.2 hRad9 is Phosphorylated in a Cell-Cycle Specific Manner

It has been established that hRad9 is phosphorylated in a cell cycle dependent manner in G2 and M phase of the cell cycle. This is the first indication of a G2 specific activity for hRad9; consistent with the data from fission

yeast indicating that Rad9 is responsible for arresting the cell at G2/M in response to DNA damage (21). If hRad9 activity is in fact regulated in a cell cycle dependent manner it would be consistent hRad9 existing in the same pathway as Chk1 as the formation of hRad9σ occurs at the same point in the cell cycle as Chk1 activity is up-regulated (151). Furthermore, it has been demonstrated that all of the hRad9 in the cell appears to associate with hHus1 (188). Given the intimate association between hRad9 and hHus1 and the fact that *hus1*$^{-/-}$ mouse embryonic fibroblasts lack Chk1, but not Chk2 phosphorylation in response to DNA damage, strongly indicates that hRad9 transduces the DNA damage signal through Chk1.

Interestingly, the cell cycle phosphorylation appears to occur only in the α-form of hRad9, indicating that hRad9 must be constitutively phosphorylated in order for cell cycle phosphorylation to occur (193). The dependency on the α-form of hRad9 is evident in the endogenous protein and in the over-expressed proteins from cells treated with nocodazole. The reason for this is unclear; however a possible explanation could be that the constitutive phosphorylation of hRad9 regulates its localization within the nucleus, or the binding to the kinase responsible for cell cycle specific phosphorylation.

The cell cycle phosphorylation of hRad9 was demonstrated to be independent and distinct of the IR inducible phosphorylation occurring at all points in the cell cycle. This indicates that while hRad9 may be phosphorylated in response to DNA damage at any time, a secondary level of regulation exists within the

context of the cell cycle, possibly directing different types of DNA repair or different cellular responses to DNA damage dependent on cell cycle phase.

Furthermore, the nature of the cell cycle phosphorylation is very interesting; the hRad9σ forms are observed independent of exogenous DNA damage during G2 and M in HeLa cells, but are not observed in hTERT-RPE1 cells unless damaged in G2/M. The fact that the cell cycle phosphorylation occurs spontaneously in a cancer cell line, and only after damage in normal cells, would be consistent with a role for hRad9 in the surveillance of DNA damage in the cell, and the G2 DNA damage checkpoint. Since cancer cells accrue mutations at a much higher rate than normal cells (237, 238), hRad9 may be phosphorylated in response to endogenously arising DNA damage in HeLa cells, whereas the phosphorylation only occurs in hTERT-RPE1 cells when exposed to exogenous sources of DNA damage. This G2/M specific hRad9 phosphorylation may be of use as a marker for elevated mutation rates or genomic instability once the sites of cell cycle phosphorylation are mapped and phospho-specific antibodies can be generated.

4.3 hRad9 Mutagenesis

Once it was demonstrated that the G2/M specific phosphorylation could be seen in heterologously expressed hRad9, a site directed mutagenesis approach was undertaken to identify the sites at which cell cycle specific phosphorylation was occurring. Unfortunately, none of the point mutants studied abrogated the cell cycle phosphorylation pattern observed in over-expressed hRad9 in

nocodazole treated cells. However, it should be noted that the identification of phosphorylation sites was done exclusively on the basis of differences in electrophoretic mobility, and the phosphorylation sites studied may indeed comprise constitutive phosphorylation sites which do not reveal obvious differences in electrophoretic mobility.

The identification of the sites of cell cycle phosphorylation may yield insight into the function of hRad9. A more practical and unbiased approach to the analysis of hRad9 cell cycle phosphorylation would involve immunoprecipitation of large quantities of hRad9 protein and the analysis of phosphorylation by tandem mass spectrometry. Identification of the sites of cell cycle phosphorylation on hRad9 would allow study of mutant proteins lacking phosphorylation sites, and the role of cell cycle phosphorylation in DNA damage sensitivity and checkpoint control could be determined.

4.3.1 Cell Cycle Effects of hRad9 Point Mutants

When overexpressed, none of the point mutants affected normal cell cycle progression or IR induced G2 arrest in the assay used. However, it should be noted that despite the mutant proteins being present at approximately a 100 fold excess compared to endogenous hRad9 (on the basis of hRad9 fluorescence) the possibility that any effect of the hRad9 mutants may not be dominant, and as a result would not be noticeable in the presence of the endogenous protein. Of the mutants made, four (S21A, S43A, S44A and S46A) corresponded to sites in the fission yeast protein, that when mutated, subtly sensitized cells to DNA

damage (236). If any effect of the mutants were subtle it may possibly not be detected in the presence of the endogenous protein, and thus to appropriately characterize the cell cycle or DNA damage sensitivity effect of any hRad9 point mutant a hRad9 null cell line will be required.

4.4 Formation of Extraction Resistant hRad9 Complexes is not Dependent on G2/M Specific Phosphorylation.

Consistent with another report it was observed that a portion of the hRad9 in the cell could be converted to an extraction resistant form, indicative of chromatin binding in response to DNA damage or replication inhibition (143, 195). The extraction resistance of hRad9, as an indication of chromatin binding, was used to determine if the cell cycle phosphorylation affected the extraction resistance in response to DNA damage at various points in the cell cycle.

The observation that the abundance of extraction resistant hRad9 was increased in all cells synchronized with thymidine to S, G2 and M phase of the cell cycle has two possibilities. Firstly, this result suggests that hRad9 is loaded onto chromatin in a cell cycle dependent manner, which would be consistent with the notion that hRad9 is responsible for genome surveillance acting at the G2 checkpoint. It is also of note that in asynchronous cells as well as cells that were synchronized to S and G2 phases of the cell cycle that the extraction resistant pool of hRad9 was phosphorylated in response to IR in a banding pattern consistent with that of hRad9σ. This suggests that chromatin bound hRad9 is phosphorylated in response to DNA damage, and that hRad9σ may be present at

other points in the cell cycle. However, it is not known whether the chromatin bound hRad9 is phosphorylated on the same residue in response to DNA damage, or by the same kinase, as that observed in the cell cycle specific hRad9σ form.

Nevertheless, the second possibility is that should be noted is that the increased abundance of extraction resistant hRad9 in S, G2 and M phases of the cell cycle could be the result of the thymidine treatment causing DNA damage in the cells that does not result in hRad9 being phosphorylated. An ideal resolution of this paradox would be the more difficult method of elutriation for generation of synchronous populations of cells to examine the interdependence between cell cycle position, hRad9 phosphorylation, and hRad9 extractability in the presence and absence of DNA damage.

Additionally, it is of note that in cells that were synchronized to mitosis by nocodazole treatment hRad9σ could be loaded onto chromatin in response to IR, suggesting that hRad9 cell cycle phosphorylation does not affect the chromatin binding of hRad9. However, this question of cell cycle phosphorylation and hRad9 extractability will not be answered rigorously without the identification of the sites of cell cycle phosphorylation and the generation of mutants lacking those sites.

4.5 Conclusion

The human Rad9 checkpoint protein has been demonstrated to exist in three complexes in cell extracts; the differences in function between the three

complexes await characterization. An important first step in the determination of the function of the three hRad9 complexes will be the identification of all other factors comprising them. Clearly, a comprehensive, unbiased analysis of hRad9 protein-protein interactions using emerging proteomic technologies would be of great value in understanding of hRad9 function.

The hRad9 protein is phosphorylated at G2/M of the cell cycle in a manner that appears to be independent of the damage induced phosphorylation attributed to the ATM kinase. If hRad9 cell cycle phosphorylation does not affect chromatin binding then the identification of the role of hRad9 cell cycle phosphorylation will be of great interest in our understanding of the role of hRad9 in the cell. It will be very interesting to see if mutation of hRad9 cell cycle phosphorylation sites has the same cell cycle effect as in the mutation hRad17 phosphorylation sites which results in the abolition of G2/M checkpoint activity (144).

Furthermore, the generation of a conditional cell line which can be deleted for hRad9 by the addition of the Cre-recombinase, like that described by Cortez *et al.* for the ATR kinase (129), would be ideal for in the screening of hRad9 point mutants for *bona fide* checkpoint defects and sensitivity to DNA damage. Furthermore, the conditional cell line would allow for the assay for the function of the hRad9 checkpoint protein similar to the powerful analyses performed in fission yeast.

Literature Cited

1. Weinert, T. and L. Hartwell, *Control of G2 delay by the rad9 gene of Saccharomyces cerevisiae.* J Cell Sci Suppl, 1989. **12**: p. 145-8.

2. Weinert, T.A. and L.H. Hartwell, *The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae.* Science, 1988. **241**(4863): p. 317-22.

3. Hartwell, L.H. and T.A. Weinert, *Checkpoints: controls that ensure the order of cell cycle events.* Science, 1989. **246**(4930): p. 629-34.

4. Elledge, S.J., *Cell cycle checkpoints: preventing an identity crisis.* Science, 1996. **274**(5293): p. 1664-72.

5. Zhou, Z. and S.J. Elledge, *DUN1 encodes a protein kinase that controls the DNA damage response in yeast.* Cell, 1993. **75**(6): p. 1119-27.

6. Kastan, M.B., O. Onyekwere, D. Sidransky, B. Vogelstein, and R.W. Craig, *Participation of p53 protein in the cellular response to DNA damage.* Cancer Res, 1991. **51**(23 Pt 1): p. 6304-11.

7. Chow, K.N., P. Starostik, and D.C. Dean, *The Rb family contains a conserved cyclin-dependent-kinase-regulated transcriptional repressor motif.* Mol Cell Biol, 1996. **16**(12): p. 7173-81.

8. el-Deiry, W.S., J.W. Harper, P.M. O'Connor, V.E. Velculescu, C.E. Canman, J. Jackman, J.A. Pietenpol, M. Burrell, D.E. Hill, Y. Wang, and et al., *WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis.* Cancer Res, 1994. **54**(5): p. 1169-74.

9. Di Leonardo, A., S.P. Linke, K. Clarkin, and G.M. Wahl, *DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts.* Genes Dev, 1994. **8**(21): p. 2540-51.

10. Findley, H.W., L. Gu, A.M. Yeager, and M. Zhou, *Expression and regulation of Bcl-2, Bcl-xl, and Bax correlate with p53 status and sensitivity to apoptosis in childhood acute lymphoblastic leukemia.* Blood, 1997. **89**(8): p. 2986-93.

11. Elledge, R.M. and W.H. Lee, *Life and death by p53.* Bioessays, 1995. **17**(11): p. 923-30.

12. Zhou, B.B. and S.J. Elledge, *The DNA damage response: putting checkpoints in perspective.* Nature, 2000. **408**(6811): p. 433-9.

13. Kastan, M.B., Q. Zhan, W.S. el-Deiry, F. Carrier, T. Jacks, W.V. Walsh, B.S. Plunkett, B. Vogelstein, and A.J. Fornace, Jr., *A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia.* Cell, 1992. **71**(4): p. 587-97.

14. Zhan, Q., I. Bae, M.B. Kastan, and A.J. Fornace, Jr., *The p53-dependent gamma-ray response of GADD45.* Cancer Res, 1994. **54**(10): p. 2755-60.

15. Kuerbitz, S.J., B.S. Plunkett, W.V. Walsh, and M.B. Kastan, *Wild-type p53 is a cell cycle checkpoint determinant following irradiation.* Proc Natl Acad Sci U S A, 1992. **89**(16): p. 7491-5.

16. Slichenmyer, W.J., W.G. Nelson, R.J. Slebos, and M.B. Kastan, *Loss of a p53-associated G1 checkpoint does not decrease cell survival following DNA damage.* Cancer Res, 1993. **53**(18): p. 4164-8.

17. Hoyt, M.A., L. Totis, and B.T. Roberts, *S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function.* Cell, 1991. **66**(3): p. 507-17.

18. Canman, C.E., A.C. Wolff, C.Y. Chen, A.J. Fornace, Jr., and M.B. Kastan, *The p53-dependent G1 cell cycle checkpoint pathway and ataxia-telangiectasia.* Cancer Res, 1994. **54**(19): p. 5054-8.

19. Xu, B., S.T. Kim, D.S. Lim, and M.B. Kastan, *Two molecularly distinct g(2)/m checkpoints are induced by ionizing irradiation.* Mol Cell Biol, 2002. **22**(4): p. 1049-59.

20. Orren, D.K., L.N. Petersen, and V.A. Bohr, *A UV-responsive G2 checkpoint in rodent cells.* Mol Cell Biol, 1995. **15**(7): p. 3722-30.

21. al-Khodairy, F. and A.M. Carr, *DNA repair mutants defining G2 checkpoint pathways in Schizosaccharomyces pombe.* Embo J, 1992. **11**(4): p. 1343-50.

22. al-Khodairy, F., E. Fotou, K.S. Sheldrick, D.J. Griffiths, A.R. Lehmann, and A.M. Carr, *Identification and characterization of new elements involved in checkpoint and feedback controls in fission yeast.* Mol Biol Cell, 1994. **5**(2): p. 147-60.

23. Enoch, T., A.M. Carr, and P. Nurse, *Fission yeast genes involved in coupling mitosis to completion of DNA replication.* Genes Dev, 1992. **6**(11): p. 2035-46.

24. Weinert, T.A. and L.H. Hartwell, *Characterization of RAD9 of Saccharomyces cerevisiae and evidence that its function acts posttranslationally in cell cycle arrest after DNA damage.* Mol Cell Biol, 1990. **10**(12): p. 6554-64.

25. Taylor, W.R. and G.R. Stark, *Regulation of the G2/M transition by p53.* Oncogene, 2001. **20**(15): p. 1803-15.

26. Boddy, M.N., B. Furnari, O. Mondesert, and P. Russell, *Replication checkpoint enforced by kinases Cds1 and Chk1.* Science, 1998. **280**(5365): p. 909-12.

27. Zeng, Y., K.C. Forbes, Z. Wu, S. Moreno, H. Piwnica-Worms, and T. Enoch, *Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1.* Nature, 1998. **395**(6701): p. 507-10.

28. Nghiem, P., P.K. Park, Y.S. Kim Ys, B.N. Desai, and S.L. Schreiber, *ATR Is Not Required for p53 Activation but Synergizes with p53 in the Replication Checkpoint.* J Biol Chem, 2002. **277**(6): p. 4428-34.

29. Lim, D.S., S.T. Kim, B. Xu, R.S. Maser, J. Lin, J.H. Petrini, and M.B. Kastan, *ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway.* Nature, 2000. **404**(6778): p. 613-7.

30. Tolmach, L.J., R.W. Jones, and P.M. Busse, *The action of caffeine on X-irradiated HeLa cells. I. Delayed inhibition of DNA synthesis.* Radiat Res, 1977. **71**(3): p. 653-65.

31. Falck, J., N. Mailand, R.G. Syljuasen, J. Bartek, and J. Lukas, *The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis.* Nature, 2001. **410**(6830): p. 842-7.

32. Weiss, R.S., T. Enoch, and P. Leder, *Inactivation of mouse Hus1 results in genomic instability and impaired responses to genotoxic stress.* Genes Dev, 2000. **14**(15): p. 1886-98.

33. Rupes, I., B.A. Webb, A. Mak, and P.G. Young, *G2/M arrest caused by actin disruption is a manifestation of the cell size checkpoint in fission yeast.* Mol Biol Cell, 2001. **12**(12): p. 3892-903.

34. Li, R. and A.W. Murray, *Feedback control of mitosis in budding yeast.* Cell, 1991. **66**(3): p. 519-31.

35. Ahmed, S. and J. Hodgkin, *MRT-2 checkpoint protein is required for germline immortality and telomere replication in C. elegans.* Nature, 2000. **403**(6766): p. 159-64.

36. Vaziri, H., M.D. West, R.C. Allsopp, T.S. Davison, Y.S. Wu, C.H. Arrowsmith, G.G. Poirier, and S. Benchimol, *ATM-dependent telomere loss in aging human diploid fibroblasts and DNA damage lead to the post-translational*

activation of p53 protein involving poly(ADP-ribose) polymerase. Embo J, 1997. **16**(19): p. 6018-33.

37. Smilenov, L.B., S.E. Morgan, W. Mellado, S.G. Sawant, M.B. Kastan, and T.K. Pandita, *Influence of ATM function on telomere metabolism.* Oncogene, 1997. **15**(22): p. 2659-65.

38. Matsuura, A., T. Naito, and F. Ishikawa, *Genetic control of telomere integrity in Schizosaccharomyces pombe: rad3(+) and tel1(+) are parts of two regulatory networks independent of the downstream protein kinases chk1(+) and cds1(+).* Genetics, 1999. **152**(4): p. 1501-12.

39. Fritz, E., A.A. Friedl, R.M. Zwacka, F. Eckardt-Schupp, and M.S. Meyn, *The yeast TEL1 gene partially substitutes for human ATM in suppressing hyperrecombination, radiation-induced apoptosis and telomere shortening in A-T cells.* Mol Biol Cell, 2000. **11**(8): p. 2605-16.

40. Kishi, S. and K.P. Lu, *A critical role for Pin2/TRF1 in ATM-dependent regulation: Inhibition of Pin2/TRF1 function complements telomere shortening, the radiosensitivity and G2/M checkpoint defect of Ataxia-Telangiectasia cells.* J Biol Chem, 2001. **13**: p. 13.

41. Murakami, H. and P. Nurse, *DNA replication and damage checkpoints and meiotic cell cycle controls in the fission and budding yeasts.* Biochem J, 2000. **349**(Pt 1): p. 1-12.

42. Dahlen, M., T. Olsson, G. Kanter-Smoler, A. Ramne, and P. Sunnerhagen, *Regulation of telomere length by checkpoint genes in Schizosaccharomyces pombe.* Mol Biol Cell, 1998. **9**(3): p. 611-21.

43. Murakami, H. and P. Nurse, *Meiotic DNA replication checkpoint control in fission yeast.* Genes Dev, 1999. **13**(19): p. 2581-93.

44. Freire, R., J.R. Murguia, M. Tarsounas, N.F. Lowndes, P.B. Moens, and S.P. Jackson, *Human and mouse homologs of Schizosaccharomyces pombe rad1(+) and Saccharomyces cerevisiae RAD17: linkage to checkpoint control and mammalian meiosis.* Genes Dev, 1998. **12**(16): p. 2560-73.

45. Hong, E.J. and G.S. Roeder, *A role for Ddc1 in signaling meiotic double-strand breaks at the pachytene checkpoint.* Genes Dev, 2002. **16**(3): p. 363-76.

46. Lydall, D., Y. Nikolsky, D.K. Bishop, and T. Weinert, *A meiotic recombination checkpoint controlled by mitotic checkpoint genes.* Nature, 1996. **383**(6603): p. 840-3.

47. Tarsounas, M. and P.B. Moens, *Checkpoint and DNA-repair proteins are associated with the cores of mammalian meiotic chromosomes.* Curr Top Dev Biol, 2001. **51**: p. 109-34.

48. Barlow, C., S. Hirotsune, R. Paylor, M. Liyanage, M. Eckhaus, F. Collins, Y. Shiloh, J.N. Crawley, T. Ried, D. Tagle, and A. Wynshaw-Boris, *Atm-deficient mice: a paradigm of ataxia telangiectasia.* Cell, 1996. **86**(1): p. 159-71.

49. Lett, J.T., I. Caldwell, C.J. Dean, and P. Alexander, *Rejoining of x-ray induced breaks in the DNA of leukaemia cells.* Nature, 1967. **214**(90): p. 790-2.

50. Ormerod, M.G. and U. Stevens, *The rejoining of x-ray-induced strand breaks in the DNA of a murine lymphoma cell (L5178Y).* Biochim Biophys Acta, 1971. **232**(1): p. 72-82.

51. Lawley, P.D., A.R. Crathorn, S.A. Shah, and B.A. Smith, *Biomethylation of deoxyribonucleic acid in cultured human tumour cells (HeLa). Methylated bases other than 5-methylcytosine not detected.* Biochem J, 1972. **128**(1): p. 133-8.

52. Loveless, A., *Possible relevance of O-6 alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides.* Nature, 1969. **223**(202): p. 206-7.

53. Beukers, R., and Berends, W., *Isolation and Identifcation of the Irradiation Product of Thymine.* Biochemica and Biophysica Acta, 1960. **41**: p. 550-551.

54. Edenberg, H.J., *Inhibition of DNA Replication by Ultraviolet Light.* Biophysical Journal, 1976. **16**: p. 849-860.

55. Yeh, Y.C. and I. Tessman, *Differential effect of hydroxyurea on a ribonucleotide reductase system.* J Biol Chem, 1978. **253**(5): p. 1323-4.

56. Tercero, J.A. and J.F. Diffley, *Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint.* Nature, 2001. **412**(6846): p. 553-7.

57. Murray, J.M., H.D. Lindsay, C.A. Munday, and A.M. Carr, *Role of Schizosaccharomyces pombe RecQ homolog, recombination, and checkpoint genes in UV damage tolerance.* Mol Cell Biol, 1997. **17**(12): p. 6868-75.

58. Davis, T.W., C. Wilson-Van Patten, M. Meyers, K.A. Kunugi, S. Cuthill, C. Reznikoff, C. Garces, C.R. Boland, T.J. Kinsella, R. Fishel, and D.A. Boothman, *Defective expression of the DNA mismatch repair protein, MLH1, alters G2-M cell cycle checkpoint arrest following ionizing radiation.* Cancer Res, 1998. **58**(4): p. 767-78.

59. Hawn, M.T., A. Umar, J.M. Carethers, G. Marra, T.A. Kunkel, C.R. Boland, and M. Koi, *Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint.* Cancer Res, 1995. **55**(17): p. 3721-5.

60. de Klein, A., M. Muijtjens, R. van Os, Y. Verhoeven, B. Smit, A.M. Carr, A.R. Lehmann, and J.H. Hoeijmakers, *Targeted disruption of the cell-cycle checkpoint gene ATR leads to early embryonic lethality in mice.* Curr Biol, 2000. **10**(8): p. 479-82.

61. Takai, H., K. Tominaga, N. Motoyama, Y.A. Minamishima, H. Nagahama, T. Tsukiyama, K. Ikeda, K. Nakayama, and M. Nakanishi, *Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice.* Genes Dev, 2000. **14**(12): p. 1439-47.

62. Tsai, L.H., E. Harlow, and M. Meyerson, *Isolation of the human cdk2 gene that encodes the cyclin A- and adenovirus E1A-associated p33 kinase.* Nature, 1991. **353**(6340): p. 174-7.

63. Meyerson, M. and E. Harlow, *Identification of G1 kinase activity for cdk6, a novel cyclin D partner.* Mol Cell Biol, 1994. **14**(3): p. 2077-86.

64. Kato, J., H. Matsushime, S.W. Hiebert, M.E. Ewen, and C.J. Sherr, *Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4.* Genes Dev, 1993. **7**(3): p. 331-42.

65. Koff, A., A. Giordano, D. Desai, K. Yamashita, J.W. Harper, S. Elledge, T. Nishimoto, D.O. Morgan, B.R. Franza, and J.M. Roberts, *Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle.* Science, 1992. **257**(5077): p. 1689-94.

66. Lees, E., B. Faha, V. Dulic, S.I. Reed, and E. Harlow, *Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner.* Genes Dev, 1992. **6**(10): p. 1874-85.

67. Akiyama, T., T. Ohuchi, S. Sumida, K. Matsumoto, and K. Toyoshima, *Phosphorylation of the retinoblastoma protein by cdk2.* Proc Natl Acad Sci U S A, 1992. **89**(17): p. 7900-4.

68. Nevins, J.R., S.P. Chellappan, M. Mudryj, S. Hiebert, S. Devoto, J. Horowitz, T. Hunter, and J. Pines, *E2F transcription factor is a target for the RB protein and the cyclin A protein.* Cold Spring Harb Symp Quant Biol, 1991. **56**: p. 157-62.

69. Chittenden, T., D.M. Livingston, and W.G. Kaelin, Jr., *RB associates with an E2F-like, sequence-specific DNA-binding protein.* Cold Spring Harb Symp Quant Biol, 1991. **56**: p. 187-95.

70. Chellappan, S.P., S. Hiebert, M. Mudryj, J.M. Horowitz, and J.R. Nevins, *The E2F transcription factor is a cellular target for the RB protein.* Cell, 1991. **65**(6): p. 1053-61.

71. Hiebert, S.W., S.P. Chellappan, J.M. Horowitz, and J.R. Nevins, *The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F.* Genes Dev, 1992. **6**(2): p. 177-85.

72. DeGregori, J., T. Kowalik, and J.R. Nevins, *Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes.* Mol Cell Biol, 1995. **15**(8): p. 4215-24.

73. Ikeda, M.A., L. Jakoi, and J.R. Nevins, *A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation.* Proc Natl Acad Sci U S A, 1996. **93**(8): p. 3215-20.

74. Fagan, R., K.J. Flint, and N. Jones, *Phosphorylation of E2F-1 modulates its interaction with the retinoblastoma gene product and the adenoviral E4 19 kDa protein.* Cell, 1994. **78**(5): p. 799-811.

75. el-Deiry, W.S., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein, *WAF1, a potential mediator of p53 tumor suppression.* Cell, 1993. **75**(4): p. 817-25.

76. Harper, J.W., G.R. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge, *The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases.* Cell, 1993. **75**(4): p. 805-16.

77. Wu, H., M. Wade, L. Krall, J. Grisham, Y. Xiong, and T. Van Dyke, *Targeted in vivo expression of the cyclin-dependent kinase inhibitor p21 halts hepatocyte cell-cycle progression, postnatal liver development and regeneration.* Genes Dev, 1996. **10**(3): p. 245-60.

78. Hollstein, M., D. Sidransky, B. Vogelstein, and C.C. Harris, *p53 mutations in human cancers.* Science, 1991. **253**(5015): p. 49-53.

79. Hollstein, M.C., L. Peri, A.M. Mandard, J.A. Welsh, R. Montesano, R.A. Metcalf, M. Bak, and C.C. Harris, *Genetic analysis of human esophageal tumors from two high incidence geographic areas: frequent p53 base substitutions and absence of ras mutations.* Cancer Res, 1991. **51**(15): p. 4102-6.

80. Santibanez-Koref, M.F., J.M. Birch, A.L. Hartley, P.H. Jones, A.W. Craft, T. Eden, D. Crowther, A.M. Kelsey, and M. Harris, *p53 germline mutations in Li-Fraumeni syndrome.* Lancet, 1991. **338**(8781): p. 1490-1.

81. Yin, Y., M.A. Tainsky, F.Z. Bischoff, L.C. Strong, and G.M. Wahl, *Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles.* Cell, 1992. **70**(6): p. 937-48.

82. Hao, M., C.A. Finlay, and G. Lozano, *A functionally inactive p53 Li-Fraumeni syndrome mutant.* Oncogene, 1993. **8**(2): p. 299-306.

83. Zhan, Q., F. Carrier, and A.J. Fornace, Jr., *Induction of cellular p53 activity by DNA-damaging agents and growth arrest.* Mol Cell Biol, 1993. **13**(7): p. 4242-50.

84. Lowe, S.W., E.M. Schmitt, S.W. Smith, B.A. Osborne, and T. Jacks, *p53 is required for radiation-induced apoptosis in mouse thymocytes.* Nature, 1993. **362**(6423): p. 847-9.

85. Shaw, P., R. Bovey, S. Tardy, R. Sahli, B. Sordat, and J. Costa, *Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line.* Proc Natl Acad Sci U S A, 1992. **89**(10): p. 4495-9.

86. Yonish-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, and M. Oren, *Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6.* Nature, 1991. **352**(6333): p. 345-7.

87. Suganuma, M., T. Kawabe, H. Hori, T. Funabiki, and T. Okamoto, *Sensitization of cancer cells to DNA damage-induced cell death by specific cell cycle G2 checkpoint abrogation.* Cancer Res, 1999. **59**(23): p. 5887-91.

88. Russell, K.J., L.W. Wiens, G.W. Demers, D.A. Galloway, S.E. Plon, and M. Groudine, *Abrogation of the G2 checkpoint results in differential radiosensitization of G1 checkpoint-deficient and G1 checkpoint-competent cells.* Cancer Res, 1995. **55**(8): p. 1639-42.

89. Sarkaria, J.N., E.C. Busby, R.S. Tibbetts, P. Roos, Y. Taya, L.M. Karnitz, and R.T. Abraham, *Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine.* Cancer Res, 1999. **59**(17): p. 4375-82.

90. Busse, P.M., S.K. Bose, R.W. Jones, and L.J. Tolmach, *The action of caffeine on X-irradiated HeLa cells. II. Synergistic lethality.* Radiat Res, 1977. **71**(3): p. 666-77.

91. Deplanque, G., J. Ceraline, M.C. Mah-Becherel, J.P. Cazenave, J.P. Bergerat, and C. Klein-Soyer, *Caffeine and the G2/M block override: a concept*

resulting from a misleading cell kinetic delay, independent of functional p53. Int J Cancer, 2001. **94**(3): p. 363-9.

92. Arion, D., L. Meijer, L. Brizuela, and D. Beach, *cdc2 is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF.* Cell, 1988. **55**(2): p. 371-8.

93. Simanis, V. and P. Nurse, *The cell cycle control gene cdc2+ of fission yeast encodes a protein kinase potentially regulated by phosphorylation.* Cell, 1986. **45**(2): p. 261-8.

94. Draetta, G. and D. Beach, *Activation of cdc2 protein kinase during mitosis in human cells: cell cycle-dependent phosphorylation and subunit rearrangement.* Cell, 1988. **54**(1): p. 17-26.

95. Draetta, G., D. Beach, and E. Moran, *Synthesis of p34, the mammalian homolog of the yeast cdc2+/CDC28 protein kinase, is stimulated during adenovirus-induced proliferation of primary baby rat kidney cells.* Oncogene, 1988. **2**(6): p. 553-7.

96. Draetta, G., H. Piwnica-Worms, D. Morrison, B. Druker, T. Roberts, and D. Beach, *Human cdc2 protein kinase is a major cell-cycle regulated tyrosine kinase substrate.* Nature, 1988. **336**(6201): p. 738-44.

97. Draetta, G., L. Brizuela, B. Moran, and D. Beach, *Regulation of the vertebrate cell cycle by the cdc2 protein kinase.* Cold Spring Harb Symp Quant Biol, 1988. **53**(Pt 1): p. 195-201.

98. Draetta, G., L. Brizuela, and D. Beach, *p34, a protein kinase involved in cell cycle regulation in eukaryotic cells.* Adv Exp Med Biol, 1988. **231**: p. 453-7.

99. Riabowol, K., G. Draetta, L. Brizuela, D. Vandre, and D. Beach, *The cdc2 kinase is a nuclear protein that is essential for mitosis in mammalian cells.* Cell, 1989. **57**(3): p. 393-401.

100. Peter, M., J. Nakagawa, M. Doree, J.C. Labbe, and E.A. Nigg, *In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase.* Cell, 1990. **61**(4): p. 591-602.

101. Yamashiro, S. and F. Matsumura, *Mitosis-specific phosphorylation of caldesmon: possible molecular mechanism of cell rounding during mitosis.* Bioessays, 1991. **13**(11): p. 563-8.

102. Blangy, A., H.A. Lane, P. d'Herin, M. Harper, M. Kress, and E.A. Nigg, *Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a*

kinesin-related motor essential for bipolar spindle formation in vivo. Cell, 1995. **83**(7): p. 1159-69.

103. Lamb, N.J., A. Fernandez, A. Watrin, J.C. Labbe, and J.C. Cavadore, *Microinjection of p34cdc2 kinase induces marked changes in cell shape, cytoskeletal organization, and chromatin structure in mammalian fibroblasts.* Cell, 1990. **60**(1): p. 151-65.

104. Draetta, G., F. Luca, J. Westendorf, L. Brizuela, J. Ruderman, and D. Beach, *Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF.* Cell, 1989. **56**(5): p. 829-38.

105. Brizuela, L., G. Draetta, and D. Beach, *p13suc1 acts in the fission yeast cell division cycle as a component of the p34cdc2 protein kinase.* Embo J, 1987. **6**(11): p. 3507-14.

106. Draetta, G., L. Brizuela, J. Potashkin, and D. Beach, *Identification of p34 and p13, human homologs of the cell cycle regulators of fission yeast encoded by cdc2+ and suc1+.* Cell, 1987. **50**(2): p. 319-25.

107. Bastians, H., L.M. Topper, G.L. Gorbsky, and J.V. Ruderman, *Cell cycle-regulated proteolysis of mitotic target proteins.* Mol Biol Cell, 1999. **10**(11): p. 3927-41.

108. Vorlaufer, E. and J.M. Peters, *Regulation of the cyclin B degradation system by an inhibitor of mitotic proteolysis.* Mol Biol Cell, 1998. **9**(7): p. 1817-31.

109. Stern, B. and P. Nurse, *Cyclin B proteolysis and the cyclin-dependent kinase inhibitor rum1p are required for pheromone-induced G1 arrest in fission yeast.* Mol Biol Cell, 1998. **9**(6): p. 1309-21.

110. Muschel, R.J., H.B. Zhang, G. Iliakis, and W.G. McKenna, *Cyclin B expression in HeLa cells during the G2 block induced by ionizing radiation.* Cancer Res, 1991. **51**(19): p. 5113-7.

111. Smits, V.A., R. Klompmaker, T. Vallenius, G. Rijksen, T.P. Makela, and R.H. Medema, *p21 inhibits Thr161 phosphorylation of Cdc2 to enforce the G2 DNA damage checkpoint.* J Biol Chem, 2000. **275**(39): p. 30638-43.

112. Kumagai, A. and W.G. Dunphy, *The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system.* Cell, 1991. **64**(5): p. 903-14.

113. Morla, A.O., G. Draetta, D. Beach, and J.Y. Wang, *Reversible tyrosine phosphorylation of cdc2: dephosphorylation accompanies activation during entry into mitosis.* Cell, 1989. **58**(1): p. 193-203.

114. Lundgren, K., N. Walworth, R. Booher, M. Dembski, M. Kirschner, and D. Beach, *mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2.* Cell, 1991. **64**(6): p. 1111-22.

115. Parker, L.L. and H. Piwnica-Worms, *Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase.* Science, 1992. **257**(5078): p. 1955-7.

116. McGowan, C.H. and P. Russell, *Human Wee1 kinase inhibits cell division by phosphorylating p34cdc2 exclusively on Tyr15.* Embo J, 1993. **12**(1): p. 75-85.

117. Park, M., H.D. Chae, J. Yun, M. Jung, Y.S. Kim, S.H. Kim, M.H. Han, and D.Y. Shin, *Constitutive activation of cyclin B1-associated cdc2 kinase overrides p53-mediated G2-M arrest.* Cancer Res, 2000. **60**(3): p. 542-5.

118. Fletcher, L., Y. Cheng, and c, *Abolishment of the Tyr-15 Inhibitory Phosphorylation Site on cdc2 Reduces the Radiation-induced G(2) Delay, Revealing a Potential Checkpoint in Early Mitosis.* Cancer Res, 2002. **62**(1): p. 241-50.

119. Kao, G.D., W.G. McKenna, and R.J. Muschel, *p34(Cdc2) kinase activity is excluded from the nucleus during the radiation-induced G(2) arrest in HeLa cells.* J Biol Chem, 1999. **274**(49): p. 34779-84.

120. Dunphy, W.G. and A. Kumagai, *The cdc25 protein contains an intrinsic phosphatase activity.* Cell, 1991. **67**(1): p. 189-96.

121. Gautier, J., M.J. Solomon, R.N. Booher, J.F. Bazan, and M.W. Kirschner, *cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2.* Cell, 1991. **67**(1): p. 197-211.

122. Lee, M.S., S. Ogg, M. Xu, L.L. Parker, D.J. Donoghue, J.L. Maller, and H. Piwnica-Worms, *cdc25+ encodes a protein phosphatase that dephosphorylates p34cdc2.* Mol Biol Cell, 1992. **3**(1): p. 73-84.

123. Izumi, T. and J.L. Maller, *Elimination of cdc2 phosphorylation sites in the cdc25 phosphatase blocks initiation of M-phase.* Mol Biol Cell, 1993. **4**(12): p. 1337-50.

124. Poon, R.Y., M.S. Chau, K. Yamashita, and T. Hunter, *The role of Cdc2 feedback loop control in the DNA damage checkpoint in mammalian cells.* Cancer Res, 1997. **57**(22): p. 5168-78.

125. Siede, W., A.S. Friedberg, and E.C. Friedberg, *RAD9-dependent G1 arrest defines a second checkpoint for damaged DNA in the cell cycle of Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 1993. **90**(17): p. 7985-9.

126. Lieberman, H.B., K.M. Hopkins, M. Nass, D. Demetrick, and S. Davey, *A human homolog of the Schizosaccharomyces pombe rad9+ checkpoint control gene.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13890-5.

127. Dean, F.B., L. Lian, and M. O'Donnell, *cDNA cloning and gene mapping of human homologs for Schizosaccharomyces pombe rad17, rad1, and hus1 and cloning of homologs from mouse, Caenorhabditis elegans, and Drosophila melanogaster.* Genomics, 1998. **54**(3): p. 424-36.

128. von Deimling, F., J.M. Scharf, T. Liehr, M. Rothe, A.R. Kelter, P. Albers, W.F. Dietrich, L.M. Kunkel, N. Wernert, and B. Wirth, *Human and mouse RAD17 genes: identification, localization, genomic structure and histological expression pattern in normal testis and seminoma.* Hum Genet, 1999. **105**(1-2): p. 17-27.

129. Cortez, D., S. Guntuku, J. Qin, and S.J. Elledge, *ATR and ATRIP: partners in checkpoint signaling.* Science, 2001. **294**(5547): p. 1713-6.

130. Rowley, R., S. Subramani, and P.G. Young, *Checkpoint controls in Schizosaccharomyces pombe: rad1.* Embo J, 1992. **11**(4): p. 1335-42.

131. Walworth, N., S. Davey, and D. Beach, *Fission yeast chk1 protein kinase links the rad checkpoint pathway to cdc2.* Nature, 1993. **363**(6427): p. 368-71.

132. Walworth, N.C. and R. Bernards, *rad-dependent response of the chk1-encoded protein kinase at the DNA damage checkpoint.* Science, 1996. **271**(5247): p. 353-6.

133. Edwards, R.J., N.J. Bentley, and A.M. Carr, *A Rad3-Rad26 complex responds to DNA damage independently of other checkpoint proteins.* Nat Cell Biol, 1999. **1**(7): p. 393-8.

134. Martinho, R.G., H.D. Lindsay, G. Flaggs, A.J. DeMaggio, M.F. Hoekstra, A.M. Carr, and N.J. Bentley, *Analysis of Rad3 and Chk1 protein kinases defines different checkpoint responses.* Embo J, 1998. **17**(24): p. 7239-49.

135. Furnari, B., N. Rhind, and P. Russell, *Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase.* Science, 1997. **277**(5331): p. 1495-7.

136. Banin, S., L. Moyal, S. Shieh, Y. Taya, C.W. Anderson, L. Chessa, N.I. Smorodinsky, C. Prives, Y. Reiss, Y. Shiloh, and Y. Ziv, *Enhanced phosphorylation of p53 by ATM in response to DNA damage.* Science, 1998. **281**(5383): p. 1674-7.

137. Tibbetts, R.S., K.M. Brumbaugh, J.M. Williams, J.N. Sarkaria, W.A. Cliby, S.Y. Shieh, Y. Taya, C. Prives, and R.T. Abraham, *A role for ATR in the DNA damage-induced phosphorylation of p53.* Genes Dev, 1999. **13**(2): p. 152-7.

138. Lakin, N.D., B.C. Hann, and S.P. Jackson, *The ataxia-telangiectasia related protein ATR mediates DNA-dependent phosphorylation of p53.* Oncogene, 1999. **18**(27): p. 3989-95.

139. Khosravi, R., R. Maya, T. Gottlieb, M. Oren, Y. Shiloh, and D. Shkedy, *Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 14973-7.

140. Maya, R., M. Balass, S.T. Kim, D. Shkedy, J.F. Leal, O. Shifman, M. Moas, T. Buschmann, Z. Ronai, Y. Shiloh, M.B. Kastan, E. Katzir, and M. Oren, *ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage.* Genes Dev, 2001. **15**(9): p. 1067-77.

141. Tibbetts, R.S., D. Cortez, K.M. Brumbaugh, R. Scully, D. Livingston, S.J. Elledge, and R.T. Abraham, *Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress.* Genes Dev, 2000. **14**(23): p. 2989-3002.

142. Cortez, D., Y. Wang, J. Qin, and S.J. Elledge, *Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks.* Science, 1999. **286**(5442): p. 1162-6.

143. Zou, L., D. Cortez, and S.J. Elledge, *Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin.* Genes Dev, 2002. **16**(2): p. 198-208.

144. Bao, S., R.S. Tibbetts, K.M. Brumbaugh, Y. Fang, D.A. Richardson, A. Ali, S.M. Chen, R.T. Abraham, and X.F. Wang, *ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses.* Nature, 2001. **411**(6840): p. 969-74.

145. Post, S., Y.C. Weng, K. Cimprich, L.B. Chen, Y. Xu, and E.Y. Lee, *Phosphorylation of serines 635 and 645 of human Rad17 is cell cycle regulated and is required for G(1)/S checkpoint activation in response to DNA damage.* Proc Natl Acad Sci U S A, 2001. **98**(23): p. 13102-7.

146. Chen, M.J., Y.T. Lin, H.B. Lieberman, G. Chen, and E.Y. Lee, *ATM-dependent phosphorylation of human Rad9 is required for ionizing radiation-induced checkpoint activation.* J Biol Chem, 2001. **276**(19): p. 16580-6.

147. Ward, I.M. and J. Chen, *Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress.* J Biol Chem, 2001. **276**(51): p. 47759-62.

148. Burma, S., B.P. Chen, M. Murphy, A. Kurimasa, and D.J. Chen, *ATM phosphorylates histone H2AX in response to DNA double-strand breaks.* J Biol Chem, 2001. **276**(45): p. 42462-7.

149. Zhao, H. and H. Piwnica-Worms, *ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1.* Mol Cell Biol, 2001. **21**(13): p. 4129-39.

150. Liu, Q., S. Guntuku, X.S. Cui, S. Matsuoka, D. Cortez, K. Tamai, G. Luo, S. Carattini-Rivera, F. DeMayo, A. Bradley, L.A. Donehower, and S.J. Elledge, *Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint.* Genes Dev, 2000. **14**(12): p. 1448-59.

151. Kaneko, Y.S., N. Watanabe, H. Morisaki, H. Akita, A. Fujimoto, K. Tominaga, M. Terasawa, A. Tachibana, K. Ikeda, M. Nakanishi, and Y. Kaneko, *Cell-cycle-dependent and ATM-independent expression of human Chk1 kinase.* Oncogene, 1999. **18**(25): p. 3673-81.

152. Guo, Z., A. Kumagai, S.X. Wang, and W.G. Dunphy, *Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in Xenopus egg extracts.* Genes Dev, 2000. **14**(21): p. 2745-56.

153. Matsuoka, S., M. Huang, and S.J. Elledge, *Linkage of ATM to cell cycle regulation by the Chk2 protein kinase.* Science, 1998. **282**(5395): p. 1893-7.

154. Chaturvedi, P., W.K. Eng, Y. Zhu, M.R. Mattern, R. Mishra, M.R. Hurle, X. Zhang, R.S. Annan, Q. Lu, L.F. Faucette, G.F. Scott, X. Li, S.A. Carr, R.K. Johnson, J.D. Winkler, and B.B. Zhou, *Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway.* Oncogene, 1999. **18**(28): p. 4047-54.

155. Brown, A.L., C.H. Lee, J.K. Schwarz, N. Mitiku, H. Piwnica-Worms, and J.H. Chung, *A human Cds1-related kinase that functions downstream of ATM protein in the cellular response to DNA damage.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3745-50.

156. Hekmat-Nejad, M., Z. You, M.C. Yee, J.W. Newport, and K.A. Cimprich, *Xenopus ATR is a replication-dependent chromatin-binding protein required for the DNA replication checkpoint.* Curr Biol, 2000. **10**(24): p. 1565-73.

157. Smith, G.C., R.B. Cary, N.D. Lakin, B.C. Hann, S.H. Teo, D.J. Chen, and S.P. Jackson, *Purification and DNA binding properties of the ataxia-telangiectasia gene product ATM.* Proc Natl Acad Sci U S A, 1999. **96**(20): p. 11134-9.

158. Kastan, M.B. and D.S. Lim, *The many substrates and functions of ATM.* Nat Rev Mol Cell Biol, 2000. **1**(3): p. 179-86.

159. Kim, S.T., D.S. Lim, C.E. Canman, and M.B. Kastan, *Substrate specificities and identification of putative substrates of ATM kinase family members.* J Biol Chem, 1999. **274**(53): p. 37538-43.

160. Zampetti-Bosseler, F. and D. Scott, *Cell death, chromosome damage and mitotic delay in normal human, ataxia telangiectasia and retinoblastoma fibroblasts after x-irradiation.* Int J Radiat Biol Relat Stud Phys Chem Med, 1981. **39**(5): p. 547-58.

161. Savitsky, K., A. Bar-Shira, S. Gilad, G. Rotman, Y. Ziv, L. Vanagaite, D.A. Tagle, S. Smith, T. Uziel, S. Sfez, and et al., *A single ataxia telangiectasia gene with a product similar to PI-3 kinase.* Science, 1995. **268**(5218): p. 1749-53.

162. Abraham, R.T., *Cell cycle checkpoint signaling through the ATM and ATR kinases.* Genes Dev, 2001. **15**(17): p. 2177-96.

163. Khanna, K.K., H. Beamish, J. Yan, K. Hobson, R. Williams, I. Dunn, and M.F. Lavin, *Nature of G1/S cell cycle checkpoint defect in ataxia-telangiectasia.* Oncogene, 1995. **11**(4): p. 609-18.

164. Chen, P., M. Gatei, M.J. O'Connell, K.K. Khanna, S.J. Bugg, A. Hogg, S.P. Scott, K. Hobson, and M.F. Lavin, *Chk1 complements the G2/M checkpoint defect and radiosensitivity of ataxia-telangiectasia cells.* Oncogene, 1999. **18**(1): p. 249-56.

165. Lavin, M., *Role of the ataxia-telangiectasia gene (ATM) in breast cancer. A-T heterozygotes seem to have an increased risk but its size is unknown.* Bmj, 1998. **317**(7157): p. 486-7.

166. Broeks, A., J.H. Urbanus, A.N. Floore, E.C. Dahler, J.G. Klijn, E.J. Rutgers, P. Devilee, N.S. Russell, F.E. van Leeuwen, and L.J. van't Veer, *ATM-heterozygous germline mutations contribute to breast cancer-susceptibility.* Am J Hum Genet, 2000. **66**(2): p. 494-500.

167. Gatti, R.A., A. Tward, and P. Concannon, *Cancer risk in ATM heterozygotes: a model of phenotypic and mechanistic differences between missense and truncating mutations.* Mol Genet Metab, 1999. **68**(4): p. 419-23.

168. Chen, P.C., M.F. Lavin, C. Kidson, and D. Moss, *Identification of ataxia telangiectasia heterozygotes, a cancer prone population.* Nature, 1978. **274**(5670): p. 484-6.

169. Angele, S. and J. Hall, *The ATM gene and breast cancer: is it really a risk factor?* Mutat Res, 2000. **462**(2-3): p. 167-78.

170. Khanna, K.K., *Cancer risk and the ATM gene: a continuing debate.* J Natl Cancer Inst, 2000. **92**(10): p. 795-802.

171. Drumea, K.C., E. Levine, J. Bernstein, B. Shank, S. Green, E. Kaplan, L. Mandell, J. Cropley, J. Obropta, I. Braccia, A. Krupnik, and B.S. Rosenstein, *ATM heterozygosity and breast cancer: screening of 37 breast cancer patients for ATM mutations using a non-isotopic RNase cleavage-based assay.* Breast Cancer Res Treat, 2000. **61**(1): p. 79-85.

172. Cliby, W.A., C.J. Roberts, K.A. Cimprich, C.M. Stringer, J.R. Lamb, S.L. Schreiber, and S.H. Friend, *Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints.* Embo J, 1998. **17**(1): p. 159-69.

173. Brown, E.J. and D. Baltimore, *ATR disruption leads to chromosomal fragmentation and early embryonic lethality.* Genes Dev, 2000. **14**(4): p. 397-402.

174. Weiss, R.S., S. Matsuoka, S.J. Elledge, and P. Leder, *Hus1 acts upstream of chk1 in a Mammalian DNA damage response pathway.* Curr Biol, 2002. **12**(1): p. 73-7.

175. Venclovas, C. and M.P. Thelen, *Structure-based predictions of Rad1, Rad9, Hus1 and Rad17 participation in sliding clamp and clamp-loading complexes.* Nucleic Acids Res, 2000. **28**(13): p. 2481-93.

176. Uhlmann, F., J. Cai, H. Flores-Rozas, F.B. Dean, J. Finkelstein, M. O'Donnell, and J. Hurwitz, *In vitro reconstitution of human replication factor C from its five subunits.* Proc Natl Acad Sci U S A, 1996. **93**(13): p. 6521-6.

177. Gomes, X.V., S.L. Schmidt, and P.M. Burgers, *ATP utilization by yeast replication factor C. II. Multiple stepwise ATP binding events are required to load proliferating cell nuclear antigen onto primed DNA.* J Biol Chem, 2001. **276**(37): p. 34776-83.

178. Gomes, X.V. and P.M. Burgers, *ATP utilization by yeast replication factor C. I. ATP-mediated interaction with DNA and with proliferating cell nuclear antigen.* J Biol Chem, 2001. **276**(37): p. 34768-75.

179. Gary Schmidt, S.L., A.L. Pautz, and P.M. Burgers, *ATP utilization by yeast replication factor C. IV. RFC ATP-binding mutants show defects in DNA replication, DNA repair and checkpoint regulation.* J Biol Chem, 2001. **29**: p. 29.

180. Kai, M., H. Tanaka, and T.S. Wang, *Fission yeast Rad17 associates with chromatin in response to aberrant genomic structures.* Mol Cell Biol, 2001. **21**(10): p. 3289-301.

181. Lindsey-Boltz, L.A., V.P. Bermudez, J. Hurwitz, and A. Sancar, *Purification and characterization of human DNA damage checkpoint Rad complexes.* Proc Natl Acad Sci U S A, 2001. **98**(20): p. 11236-41.

182. Wang, X., L. Wang, M.D. Callister, J.B. Putnam, L. Mao, and L. Li, *Human Rad17 is phosphorylated upon DNA damage and also overexpressed in primary non-small cell lung cancer tissues.* Cancer Res, 2001. **61**(20): p. 7417-21.

183. Rauen, M., M.A. Burtelow, V.M. Dufault, and L.M. Karnitz, *The human checkpoint protein hRad17 interacts with the PCNA-like proteins hRad1, hHus1, and hRad9.* J Biol Chem, 2000. **275**(38): p. 29767-71.

184. Bessho, T. and A. Sancar, *Human DNA damage checkpoint protein hRAD9 is a 3' to 5' exonuclease.* J Biol Chem, 2000. **275**(11): p. 7451-4.

185. Parker, A.E., I. Van de Weyer, M.C. Laus, I. Oostveen, J. Yon, P. Verhasselt, and W.H. Luyten, *A human homologue of the Schizosaccharomyces pombe rad1+ checkpoint gene encodes an exonuclease.* J Biol Chem, 1998. **273**(29): p. 18332-9.

186. St Onge, R.P., C.M. Udell, R. Casselman, and S. Davey, *The human G2 checkpoint control protein hRAD9 is a nuclear phosphoprotein that forms complexes with hRAD1 and hHUS1.* Mol Biol Cell, 1999. **10**(6): p. 1985-95.

187. Volkmer, E. and L.M. Karnitz, *Human homologs of Schizosaccharomyces pombe rad1, hus1, and rad9 form a DNA damage-responsive protein complex.* J Biol Chem, 1999. **274**(2): p. 567-70.

188. Burtelow, M.A., P.M. Roos-Mattjus, M. Rauen, J.R. Babendure, and L.M. Karnitz, *Reconstitution and molecular analysis of the hRad9-hHus1-hRad1 (9-1-1) DNA damage responsive checkpoint complex.* J Biol Chem, 2001. **276**(28): p. 25903-9.

189. Kaur, R., C.F. Kostrub, and T. Enoch, *Structure-function analysis of fission yeast hus1-rad1-rad9 checkpoint complex.* Mol Biol Cell, 2001. **12**(12): p. 3744-58.

190. Cai, R.L., Y. Yan-Neale, M.A. Cueto, H. Xu, and D. Cohen, *HDAC1, a histone deacetylase, forms a complex with Hus1 and Rad9, two G2/M checkpoint Rad proteins.* J Biol Chem, 2000. **275**(36): p. 27909-16.

191. Makiniemi, M., T. Hillukkala, J. Tuusa, K. Reini, M. Vaara, D. Huang, H. Pospiech, I. Majuri, T. Westerling, T.P. Makela, and J.E. Syvaoja, *BRCT domain-containing protein TopBP1 functions in DNA replication and damage response.* J Biol Chem, 2001. **276**(32): p. 30399-406.

192. Komatsu, K., T. Miyashita, H. Hang, K.M. Hopkins, W. Zheng, S. Cuddeback, M. Yamada, H.B. Lieberman, and H.G. Wang, *Human homologue of S. pombe Rad9 interacts with BCL-2/BCL-xL and promotes apoptosis.* Nat Cell Biol, 2000. **2**(1): p. 1-6.

193. St Onge, R.P., B.D. Besley, M. Park, R. Casselman, and S. Davey, *DNA damage-dependent and -independent phosphorylation of the hRad9 checkpoint protein.* J Biol Chem, 2001. **276**(45): p. 41898-905.

194. Komatsu, K., W. Wharton, H. Hang, C. Wu, S. Singh, H.B. Lieberman, W.J. Pledger, and H.G. Wang, *PCNA interacts with hHus1/hRad9 in response to DNA damage and replication inhibition.* Oncogene, 2000. **19**(46): p. 5291-7.

195. Burtelow, M.A., S.H. Kaufmann, and L.M. Karnitz, *Retention of the human Rad9 checkpoint complex in extraction-resistant nuclear complexes after DNA damage.* J Biol Chem, 2000. **275**(34): p. 26343-8.

196. Parker, A.E., I. Van de Weyer, M.C. Laus, P. Verhasselt, and W.H. Luyten, *Identification of a human homologue of the Schizosaccharomyces pombe rad17+ checkpoint gene.* J Biol Chem, 1998. **273**(29): p. 18340-6.

197. O'Connell, M.J., J.M. Raleigh, H.M. Verkade, and P. Nurse, *Chk1 is a wee1 kinase in the G2 DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation.* Embo J, 1997. **16**(3): p. 545-54.

198. Sanchez, Y., C. Wong, R.S. Thoma, R. Richman, Z. Wu, H. Piwnica-Worms, and S.J. Elledge, *Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25.* Science, 1997. **277**(5331): p. 1497-501.

199. Peng, C.Y., P.R. Graves, R.S. Thoma, Z. Wu, A.S. Shaw, and H. Piwnica-Worms, *Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216.* Science, 1997. **277**(5331): p. 1501-5.

200. Baber-Furnari, B.A., N. Rhind, M.N. Boddy, P. Shanahan, A. Lopez-Girona, and P. Russell, *Regulation of mitotic inhibitor Mik1 helps to enforce the DNA damage checkpoint.* Mol Biol Cell, 2000. **11**(1): p. 1-11.

201. Christensen, P.U., N.J. Bentley, R.G. Martinho, O. Nielsen, and A.M. Carr, *Mik1 levels accumulate in S phase and may mediate an intrinsic link between S phase and mitosis.* Proc Natl Acad Sci U S A, 2000. **97**(6): p. 2579-84.

202. Furnari, B., A. Blasina, M.N. Boddy, C.H. McGowan, and P. Russell, *Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1.* Mol Biol Cell, 1999. **10**(4): p. 833-45.

203. Blasina, A., I.V. de Weyer, M.C. Laus, W.H. Luyten, A.E. Parker, and C.H. McGowan, *A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase.* Curr Biol, 1999. **9**(1): p. 1-10.

204. Raleigh, J.M. and M.J. O'Connell, *The G(2) DNA damage checkpoint targets both Wee1 and Cdc25.* J Cell Sci, 2000. **113**(Pt 10): p. 1727-36.

205. Conklin, D.S., K. Galaktionov, and D. Beach, *14-3-3 proteins associate with cdc25 phosphatases.* Proc Natl Acad Sci U S A, 1995. **92**(17): p. 7892-6.

206. Kumagai, A. and W.G. Dunphy, *Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25.* Genes Dev, 1999. **13**(9): p. 1067-72.

207. Lopez-Girona, A., B. Furnari, O. Mondesert, and P. Russell, *Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein.* Nature, 1999. **397**(6715): p. 172-5.

208. Chan, T.A., H. Hermeking, C. Lengauer, K.W. Kinzler, and B. Vogelstein, *14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage.* Nature, 1999. **401**(6753): p. 616-20.

209. Chen, L., T.H. Liu, and N.C. Walworth, *Association of Chk1 with 14-3-3 proteins is stimulated by DNA damage.* Genes Dev, 1999. **13**(6): p. 675-85.

210. Dalal, S.N., C.M. Schweitzer, J. Gan, and J.A. DeCaprio, *Cytoplasmic localization of human cdc25C during interphase requires an intact 14-3-3 binding site.* Mol Cell Biol, 1999. **19**(6): p. 4465-79.

211. Morris, M.C., A. Heitz, J. Mery, F. Heitz, and G. Divita, *An essential phosphorylation-site domain of human cdc25C interacts with both 14-3-3 and cyclins.* J Biol Chem, 2000. **275**(37): p. 28849-57.

212. Graves, P.R., C.M. Lovly, G.L. Uy, and H. Piwnica-Worms, *Localization of human Cdc25C is regulated both by nuclear export and 14-3-3 protein binding.* Oncogene, 2001. **20**(15): p. 1839-51.

213. Honda, R., Y. Ohba, and H. Yasuda, *14-3-3 zeta protein binds to the carboxyl half of mouse wee1 kinase.* Biochem Biophys Res Commun, 1997. **230**(2): p. 262-5.

214. Lee, J., A. Kumagai, and W.G. Dunphy, *Positive regulation of Wee1 by Chk1 and 14-3-3 proteins.* Mol Biol Cell, 2001. **12**(3): p. 551-63.

215. Rothblum-Oviatt, C.J., C.E. Ryan, and H. Piwnica-Worms, *14-3-3 binding regulates catalytic activity of human wee1 kinase.* Cell Growth Differ, 2001. **12**(12): p. 581-9.

216. Hirao, A., Y.Y. Kong, S. Matsuoka, A. Wakeham, J. Ruland, H. Yoshida, D. Liu, S.J. Elledge, and T.W. Mak, *DNA damage-induced activation of p53 by the checkpoint kinase Chk2.* Science, 2000. **287**(5459): p. 1824-7.

217. Shieh, S.Y., J. Ahn, K. Tamai, Y. Taya, and C. Prives, *The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites.* Genes Dev, 2000. **14**(3): p. 289-300.

218. Chehab, N.H., A. Malikzay, M. Appel, and T.D. Halazonetis, *Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53.* Genes Dev, 2000. **14**(3): p. 278-88.

219. Lee, S.B., S.H. Kim, D.W. Bell, D.C. Wahrer, T.A. Schiripo, M.M. Jorczak, D.C. Sgroi, J.E. Garber, F.P. Li, K.E. Nichols, J.M. Varley, A.K. Godwin, K.M. Shannon, E. Harlow, and D.A. Haber, *Destabilization of CHK2 by a missense mutation associated with Li-Fraumeni Syndrome.* Cancer Res, 2001. **61**(22): p. 8062-7.

220. Melchionna, R., X.B. Chen, A. Blasina, and C.H. McGowan, *Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1.* Nat Cell Biol, 2000. **2**(10): p. 762-5.

221. Ahn, J.Y., J.K. Schwarz, H. Piwnica-Worms, and C.E. Canman, *Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation.* Cancer Res, 2000. **60**(21): p. 5934-6.

222. Tanaka, K., M.N. Boddy, X.B. Chen, C.H. McGowan, and P. Russell, *Threonine-11, phosphorylated by Rad3 and atm in vitro, is required for activation of fission yeast checkpoint kinase Cds1.* Mol Cell Biol, 2001. **21**(10): p. 3398-404.

223. Lopez-Girona, A., K. Tanaka, X.B. Chen, B.A. Baber, C.H. McGowan, and P. Russell, *Serine-345 is required for Rad3-dependent phosphorylation and function of checkpoint kinase Chk1 in fission yeast.* Proc Natl Acad Sci U S A, 2001. **98**(20): p. 11289-94.

224. Murakami, H. and H. Okayama, *A kinase from fission yeast responsible for blocking mitosis in S phase.* Nature, 1995. **374**(6525): p. 817-9.

225. Bullock, W.O., Fernandez, J. M., and Short, J. M., *XL-1 Blue: a high efficiency transforming recA Escherichia colistrain with beta-galactosidase selection.* BioTechniques, 1987. **5**: p. 376-378.

226. Clontech, *Transformer Site Directed Mutagenesis Kit User Manual*, . 2001: Palo Alto, CA.

227. Yee, C., I. Krishnan-Hewlett, C.C. Baker, R. Schlegel, and P.M. Howley, *Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines.* Am J Pathol, 1985. **119**(3): p. 361-6.

228. Gey, G.O., Coffman, W. D., and Kubicek, M. T., *Tissue Culture Studies of the Proliferative Capacity of Cervical Carcinoma and Normal Epithelilum.* Cancer Research, 1952. **12**: p. 264-265.

229. Yang, J., E. Chang, A.M. Cherry, C.D. Bangs, Y. Oei, A. Bodnar, A. Bronstein, C.P. Chiu, and G.S. Herron, *Human endothelial cell life extension by telomerase expression.* J Biol Chem, 1999. **274**(37): p. 26141-8.

230. Jiang, X.R., G. Jimenez, E. Chang, M. Frolkis, B. Kusler, M. Sage, M. Beeche, A.G. Bodnar, G.M. Wahl, T.D. Tlsty, and C.P. Chiu, *Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype.* Nat Genet, 1999. **21**(1): p. 111-4.

231. Hittelman, W.N. and P.N. Rao, *Bleomycin-induced damage in prematurely condensed chromosomes and its relationship to cell cycle progression in CHO cells.* Cancer Res, 1974. **34**(12): p. 3433-9.

232. Knehr, M., M. Poppe, M. Enulescu, W. Eickelbaum, M. Stoehr, D. Schroeter, and N. Paweletz, *A critical appraisal of synchronization methods applied to achieve maximal enrichment of HeLa cells in specific cell cycle phases.* Exp Cell Res, 1995. **217**(2): p. 546-53.

233. Takebe, Y., M. Seiki, J. Fujisawa, P. Hoy, K. Yokota, K. Arai, M. Yoshida, and N. Arai, *SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat.* Mol Cell Biol, 1988. **8**(1): p. 466-72.

234. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(259): p. 680-5.

235. Blom, N., S. Gammeltoft, and S. Brunak, *Sequence and structure-based prediction of eukaryotic protein phosphorylation sites.* J Mol Biol, 1999. **294**(5): p. 1351-62.

236. Hang, H., S.J. Rauth, K.M. Hopkins, and H.B. Lieberman, *Mutant alleles of Schizosaccharomyces pombe rad9(+) alter hydroxyurea resistance, radioresistance and checkpoint control.* Nucleic Acids Res, 2000. **28**(21): p. 4340-9.

237. Sager, R., *Mutation rates and mutational spectra in tumorigenic cell lines.* Cancer Surv, 1988. **7**(2): p. 325-33.

238. Warren, S.T., R.A. Schultz, C.C. Chang, M.H. Wade, and J.E. Trosko, *Elevated spontaneous mutation rate in Bloom syndrome fibroblasts.* Proc Natl Acad Sci U S A, 1981. **78**(5): p. 3133-7.

Blair David Alexander Besley

RR#4 Brockville, ON
K6V 5T4
(613) 342 1079
blair_besley@hotmail.com

Biographical Data

PII Redacted [redacted]

Education

Master of Science, March 2002
Queen's University, Dept of Biochemistry and Cancer Research Laboratories
Supervisor: Dr. Scott K. Davey
MSc. Thesis Entitled: Characterization of the Cell-Cycle Dependent and Independent Phosphorylation of the Human Rad9 Checkpoint Protein.

Bachelor of Science (Honours, First Class Standing), May 1999
Queen's University
Subject of Specialization in Biochemistry
4th Year Thesis Entitled: Cloning, Expression and Purification of the KringleV domain of Apolipoprotein(A).

Awards and Honours

September 2000 – R. Samuel McLaughlin Fellowship
September 2000 – Queen's Graduate Award
October 1999 – U.S Army Breast Cancer Studentship
September 1999 -- Queen's Graduate Award
January 1999 – Toronto Dominion Bank Higher Education Award
October 1995 – Royal Canadian Legion Award
June 1995 – Jim Coode Memorial Scholarship.

Publications and Scholarly Works

Publications

St Onge RP, **Besley BDA**, Park M, Casselman R, Davey S. DNA damage-dependent and -independent phosphorylation of the hRad9 checkpoint protein. *Journal of Biological Chemistry.* 2001; **276(45)**:41898-905.

Abstracts Published at Meetings

Besley BDA, St. Onge RP, Park M, Davey SK. Cell Cycle Dependent and Independent Phosphorylation of Human Rad9. Proceedings of the Fourth Annual Meeting for Basic and Clinical Research Trainees in the Faculty of Health Sciences.
Queen's University, Kingston ON

St. Onge RP, **Besley BDA**, Davey SK. HRAD9 Phosphorylation and the DNA Damage Response. Proceedings of the Fourth Annual Meeting for Basic and Clinical Research Trainees in the Faculty of Health Sciences.
Queen's University, Kingston ON

Besley BDA, St. Onge RP, Greer DA, Udell CM, and Davey SK. Characterization and Disruption of the G2 Checkpoint Complex and the DNA Damage Dependent Interaction. Era of Hope Department of Defense Breast Cancer Research Program Meeting -- Proceedings.
Atlanta GA, USA

Besley BDA, Casselman R, Davey SK. Purification of Baculovirus Expressed G2 Checkpoint Complex. Proceedings of the Third Annual Meeting for Basic and Clinical Research Trainees in the Faculty of Health Sciences.
Queen's University, Kingston ON

Employment History

Text Editor, December 2001 -- Ongoing
Textcheck Scientific Editing Service, Toronto ON
Read and corrected English in manuscripts of scientific papers submitted to editing service for publication in academic journals. Customers spoke English as a second language, typically submitting articles for publication in English language journals.

Summer Research Student, May -- August, 1998
Queen's University Dept of Biochemistry, Kingston ON
Supervisor: Dr. Peter Davies
I was involved in the systematic mutational analysis of the Spruce Budworm antifreeze protein, in addition to sequence analysis of the Shorthorn Sculpin antifreeze protein.

Summer Research Student, June -- August, 1997
University of Notre Dame Dept of Chemistry and Biochemistry, Notre Dame IN
Supervisor: Dr. Francis J. Castellino
I was responsible for carrying out the organic synthesis of FMOC-γ-carboxyglutamate in addition to the synthesis of synthetic peptides.